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6902 - BIOCHEMICAL SPECIAL INVESTIGATIONS

1985 ANNUAL REPORT

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KEYWORDS: Tobacco-specific nitrosamines (TSNA), minor alkaloids, nicotine, nitrate, nitrite, nitrate reductase, air-curing, flue-curing, ascorbic acid, tocophorol, burley, bright, V446, leaf microorganisms, HPLC, GC/TEA, bound nitrite, sterile tobacco

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SUMMARY

The project objectives are:(1) To determine the chemical, biological and physical factors which affect the formation of tobacco-specific nitrosamines (TSNA) in tobacco during curing and processing; and (2) to investigate ways to reduce or eliminate the formation of TSNA in tobacco during curing and/or processing.

Procedures were developed for minor alkaloids/nicotine, nitrate/nitrite, and nitrate reductase activity (NRA), in order to facilitate following these parameters in tobacco during curing and relate them to TSNA formation. These procedures and others already in existence were used in three curing studies planned to investigate the time-course of TSNA formation.

When V446 tobacco (a nornicotine converter strain) was air-cured, nitrate and TSNA increased in a nearly linear fashion with time. Nornicotine increased during curing in upper-stalk leaves, decreased in lower-stalk leaves, and was nearly unchanged in mid-stalk leaves, whereas nicotine decreased dramatically in upper- and mid-stalk and slightly in low-stalk. The ratios of NNN to the other TSNA were much higher than in normal strains, indicating that nornicotine is much more easily converted to NNN than is nicotine. TSNA were highest in low-stalk leaves, followed by mid-stalk, then upper-stalk. Major proteolytic activity took place in the first and second week of curing. No NRA was detected, and no nitrite was found.

During flue-curing of a very low nitrate bright tobacco, very low levels of TSNA were formed. The ratios of NNK to NNN and NAT were much lower than normal for flue-cured tobacco. Treatment with ascorbic acid and tocophorol

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esters before curing gave no noticeable change in TSNA formation. No NRA was found and no nitrite was detected.

When field-grown burley tobacco was air-cured, no NRA or nitrite was detected, and proteolytic activity was complete after the first week. Bacteria did not increase during curing, but nitrate showed a considerable increase, probably due to migration from the stalk. Alkaloids changed little during curing, but TSNA increased in a manner that was correlated with the increase in nitrate. TSNA were three times as high in lower and mid-stalk leaves after curing than in upper-stalk leaves. Ascorbyl palmitate and tocophoryl acetate caused some lowering of NNN and NAT in lower and mid-stalk leaves.

Several types of bacteria were isolated from green field-grown bright tobacco and tested in a model system for ability to reduce nitrate and effect formation of TSNA. One culture converted nitrate to nitrite efficiently and produced high levels of TSNA, but did not grow appreciably, indicating the possibility that tobacco microorganisms may not have to increase in population to influence TSNA formation. Alkaligenes denitrificans was shown to be capable of removing nitrate from tobacco extracts and thus preventing TSNA formation if pH levels were above 6.0.

Attempts were made to sterillize tobacco leaves and cure them to determine whether bacteria are important in curing with respect to TSNA formation. Although both chemical and gamma-irradiation techniques were used, reliable sterilization methods were not achieved.

Nitrosation studies were carried out under ambient conditions on both synthetic and commercial nicotine in buffers of varying pH, and the relative

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yields of NNN,NNA,NNK and NAT were measured. NAT was formed only from commercial nicotine, and was due to a small amount of anatabine present. Nornicotine was produced during nitrosation of nicotine. This was interpreted as being a hydrolysis product of an iminium ion formed during the reaction. Ratios of the individual TSNA changed markedly with small changes in pH.

Time-course studies of nicotine and nornicotine nitrosation over a 10 day period were carried out. For nornicotine, after a faster initial rate, NNN increased at a linear rate for 10 days. Nicotine nitrosation gave a nearly linear increase for NNA, but the curves for NNN and NNK showed the same trend as observed for NNN from nornicotine, indicating a difference in mechanism of formation for NNA as compared to NNN and NNK.

Experiments were performed in order to determine whether constituents of tobacco waxes are capable of binding nitrite and later transferring the -NO function to amines. Chloroform washings of tobacco leaves were nitrosated with nitrite, and the nitrosated washings allowed to react with a secondary amine in ethyl acetate solution. Positive results were obtained when the mixtures were analyzed for the corresponding nitrosamine. This suggests it is indeed possible for nitrite present during plant growth to be organically bound and transferred to amines during curing.

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ABBREVIATIONS

ANAT	Anatabine
AR	Analytical Research (Division)
C-18	Octadecasilyl-bonded silica
COT	Cotinine
DMPip	2,6-Dimethylpiperidine
GC	Gas Chromatography, Gas Chromatograph
HLC	Homogenized Leaf Curing
HPLC	High Performance Liquid Chromatography
MYOS	Myosmine
NAB	N'-Nitrosoanabasine
NADH	β-Nicotinamide Adenine Dinucleotide, Reduced Form
NAT	N'-Nitrosoanatabine
NDMPip	N-Nitroso-2,6-dimethylpiperidine
N-2-EtPip	N-Nitroso-2-ethylpiperidine
NIC	Nicotine
nm	Nanometers
NNA	4-(N-Methyl-N-nitrosamino)-4-(3-pyridyl)butanal
NNK	4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)butanone
NNN	N'-Nitrosonornicotine
NO_3^--N	Nitrate-Nitrogen
NO_2^--N	Nitrite-Nitrogen
NORN	Nornicotine
NR	Nitrate Reductase
NRA	Nitrate Reductase Activity
ODS	Octadecyasilyl-treated Silica

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OV	Oven Volatiles
RP	Reversed Phase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEA	Thermal Energy Analyzer
THF	Tetrahydrofuran
TSNA	Tobacco Specific Nitrosamines
UV	Ultraviolet

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I. OBJECTIVES

The objectives of the project during the past year were as follows:

1. To determine the chemical, biological and physical factors which affect the formation of tobacco-specific nitrosamines (TSNA) in tobacco during curing and processing.
2. To investigate ways to reduce or eliminate the formation of TSNA in tobacco during curing and/or processing.

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II. PROJECT PERSONNEL

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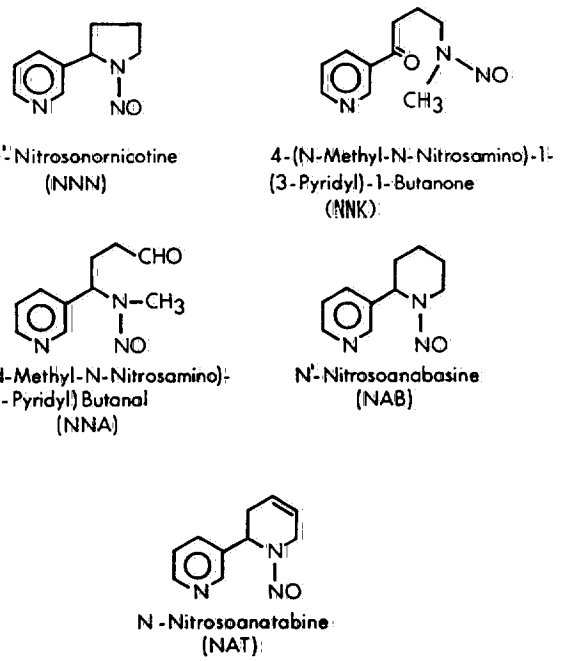
III. FORMATION OF TSNA IN FILLER

A. BACKGROUND

TSNA are nitrosamines found in cured or aged tobacco. They represent nitrosated tobacco alkaloids, as shown in Figure 1. Since TSNA are only detected after the tobacco is cured, it is generally concluded that they are formed during curing and subsequent processing. The precursors are believed to be nitrate and tobacco alkaloids, although these are not necessarily the immediate precursors.

FIGURE 1
STRUCTURES OF TOBACCO-SPECIFIC NITROSAMINES



Amines do not react with nitrate to form nitrosamines under ambient conditions; therefore, it is assumed that nitrate must be reduced to nitrite by some means before reaction can occur. During plant growth, nitrate reductase(NR) produces nitrite, which is then further reduced to ammonia and incorporated into protein. Bacteria found on the leaves also contain NR. Either of these two sources of NR could be a source of nitrite for production of TSNA.

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The nitrosating agent(NSA) may be an inorganic species derived from nitrite itself, or an organic compound formed by reaction of nitrite with a tobacco constituent. The latter would constitute a bound form of nitrite. Examples of this type of compound are nitrite esters and C-nitroso compounds. These could be formed from terpene alcohols or unsaturated compounds. Organically bound nitrite could nitrosate amines in a lipid or resin vehicle that may exist in the leaf at some stage. Inorganic (ionic) nitrite-derived NSA are more likely to nitrosate amines in an aqueous phase, and the reaction is pH dependant.

The above reactions occur by electrophilic substitution. An alternative mechanism may be the generation of a free-radical species from nitrate or nitrite, which attacks the N' position of the alkaloid, generating a radical species, which then could combine with nitrogen oxide. Unpaired electrons are known to be present in tobacco during curing, and would be expected to be accelerated by light, metal ions, or elevated temperatures such as those reached during flue-curing. Unfortunately, little is known about free radical nitrosation of tobacco alkaloids.

NNN and NAT are known to be formed when the secondary amines nornicotine and anatabine, respectively, are nitrosated. Both NNN and NNK are formed when nicotine is nitrosated, but the yields are much lower than those obtained from the reactions of the secondary amines. Nevertheless, it is generally believed that nicotine, because of the large excess present in tobacco, is the major source of NNN. This belief originated from radiochemical studies published by Hecht et al.[1], who determined that conversion efficiencies for the two alkaloids to NNN were similar under the conditions of the studies. For this to be the case, it would be necessary to propose some kind of biological intervention rather than simple chemical nitrosation. For example, nicotine

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could be converted to nornicotine by a demethylase enzyme, and then nitrosated.

In the previous Annual Report[2], a number of model studies of TSNA formation were described, in which selected possibilities were explored. It was shown that under aqueous nitrosation conditions, NAT is formed more readily from anatabine than NNN is formed from nornicotine, and that both of these reactions occur much more readily than nitrosation of nicotine. Bacteria isolated from tobacco were shown to be capable of causing nitrate reduction and TSNA formation when incubated with filter-sterilized aqueous green tobacco extracts. In some cases, bacteria were also observed to reduce nitrate without accumulation of nitrite, with little TSNA formation. There was some evidence that NRA was still at least partially intact in mature green burley; i.e., when crude filtrates containing green solid material believed to include the chloroplasts were incubated, nitrate reduction occurred, an increase in nitrite concentration was measured, and large increases in TSNA were found.

A problem with the model studies was that they were carried out in aqueous solution. It has been observed in curing studies that the major accumulation of TSNA occurs when most of the water has been lost from the leaves during curing. Another problem was that the cell structure of the leaves was disrupted during the model studies. This allowed interaction between compounds which may not be in contact during normal curing of the leaves. Also, microorganisms were able to come in contact with substrates much more easily in the slurries or extracts of the disrupted leaves. Therefore, the model studies can only be said to reveal potential pathways that may exist if conditions are favorable. For this reason, further work on the model systems was not continued this year except for some studies of the behavior of microorganisms, where the tobacco extracts were a convenient medium to assess the potential for TSNA formation and nitrate reduction.

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Air-curing experiments where TSNA have been measured have been carried out on a number of occasions during the past several years here at PM, as well as elsewhere. Sample-to-sample variation has made interpretation of the results difficult in most of these studies. However, several general conclusions can be made from the available data. TSNA levels tend to increase with increasing curing time, and the largest increases occur after the OV reaches equilibrium levels. Higher nitrate levels tend to give higher levels of TSNA, and very low levels of nitrate lead to very low TSNA levels. Also, concentrations of nitrate are highest in the midrib and at lower stalk positions, and TSNA levels tend to be highest in plant tissue where nitrate is highest. Both levels and relative populations of leaf microrganisms tend to remain fairly constant during air-curing.

In a recent publication by workers at the University of Kentucky[3], results of a comprehensive burley curing study were reported. Attempts were made to correlate nitrate levels (achieved by variations in N-fertilization) with TSNA formation, but significant correlations were not obtained. On the other hand, significant correlations were obtained between nicotine and each of the individual TSNA, and between nornicotine and TSNA. In the latter case, nornicotine was more strongly correlated with NNN than was nicotine. Anatabine concentration was strongly correlated with nicotine, indicating that anatabine is correlated with NAT, although this correlation was not measured directly. Measurements of nitrite concentration were made as well, but there was no consistent relationship between these values and TSNA levels. This was believed to be due to possible microbial reduction, eliminating any accumulation of nitrite. It was stated that the rate of nitrite formation is probably the limiting step in TSNA synthesis.

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Numerous other curing studies have also been reported in which TSNA results have been obtained. Relationships were established between TSNA and various chemical and physical factors. In one paper, a negative correlation of NNK with nicotine concentration was reported during HLC of burley tobacco, and increased shading of the lamina was found to result in higher NNK levels[4]. Higher levels of nornicotine gave higher NNN values in N. rustica after curing, and sun drying led to large increases in NNN, NAT and NNK in N. rustica and N. tabaccum[5].

In contrast to the results obtained in burley air-curing studies, no correlation was found between alkaloids and NNN in bright leaves after flue-curing[6]. No correlation was obtained between nicotine:nornicotine ratios and NNN in another flue-curing study[7], but later harvest dates led to higher levels of NNN.

Although there have been several studies of TSNA formation during curing, very little work has been reported on attempts to reduce TSNA formation. In one report, spraying of ascorbic acid and α-tocophorol (vitamin E) on the leaf surfaces before flue-curing caused a reduction in the levels of NNN in the cured leaves by 20-80%[7]. In a later report, however, an attempt to repeat this study was unsuccessful[8]. In the latter attempt, the chemicals were applied to the green plants before harvest as opposed to the earlier study where they were applied after harvest but before curing, suggesting that exposure to sunlight may have contributed to the results obtained.

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B. RESEARCH

The major focus for the research program carried out during 1985 in Project 6902 was observation of chemical and biological changes potentially related to TSNA formation during curing of tobacco. In order to carry out this research, it was necessary to develop a number of methods and procedures. Methodology for sampling and handling the tobacco was developed in order to reduce sample variation. A new method for nitrate/nitrite analysis was developed, as was a new minor alkaloid/nicotine analysis. Procedures for carrying out NRA determinations were also worked out. Since this method development was critical to the success of the studies, and because of the amount of time required, brief accounts of the work are included in this report.

Studies of alkaloid nitrosation under aqueous conditions were continued from 1984. The effect of pH changes on products of nicotine nitrosation were studied, and time-course studies of nicotine and nornicotine nitrosation were carried out. Studies of the potential nitrosation of amines in tobacco by organic NSA formed from nitrite and components of the leaf resins were also carried out.

The role of microorganisms in curing was investigated by attempting to sterilize tobacco before curing, and by isolating bacteria from green leaf and testing them for ability to reduce nitrate and produce TSNA in model systems. The role of tobacco-derived NR was also examined by measuring activity both in the growing plants and during curing.

Three curing studies were carried out. Coker 319 was flue-cured; Ky 14 and V446 (a nornicotine-converter strain) were air-cured. Data obtained from these studies are presented and discussed.

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1. Methodology

a. Nitrate/Nitrite Method

The HPLC method used for measurement of nitrate and nitrite in 1984 involved use of an ODS column and an aqueous buffer containing tetramethylammonium ion as an ion-pairing agent for the anions. Problems with this system were encountered. The buffer tended to become contaminated and had to be refrigerated, and frequent clogging of the system occurred. Modifications were made to circumvent these problems. Tetrapentylammonium ion was substituted as the ion-pairing agent, which increased retention of the ion-pairs on the column. This allowed the addition of acetonitrile to the solvent, providing more control over retention times. The acetonitrile also prevented contamination and clogging. No refrigeration of the solvent was needed, and the system could remain equilibrated for long periods of time, saving valuable time.

Other changes to the procedure were the addition of an autosampler and a computing integrator. These additions further improved reliability and speed. An internal standard (potassium iodide) also enhanced precision and accuracy.

A new extraction and sample cleanup method before the analysis was also developed. In the current procedure, a 1 g sample of ground tobacco is shaken with 10 ml of water for 30 min., centrifuged, and the extract passed through a C-18 cartridge with attached filter before placing in autosampler vials. The autosampler and HPLC are controlled by the integrator, and the method allows automatic recalibration with standard solution at regular intervals. The nitrate and nitrite values are reported directly in % NO_3^--N and NO_2^--N.

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FIGURE 2

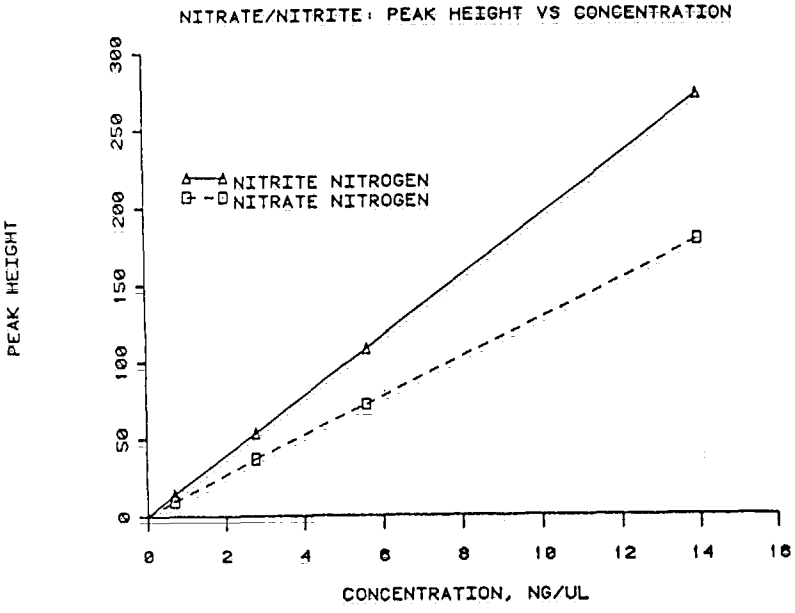


FIGURE 3

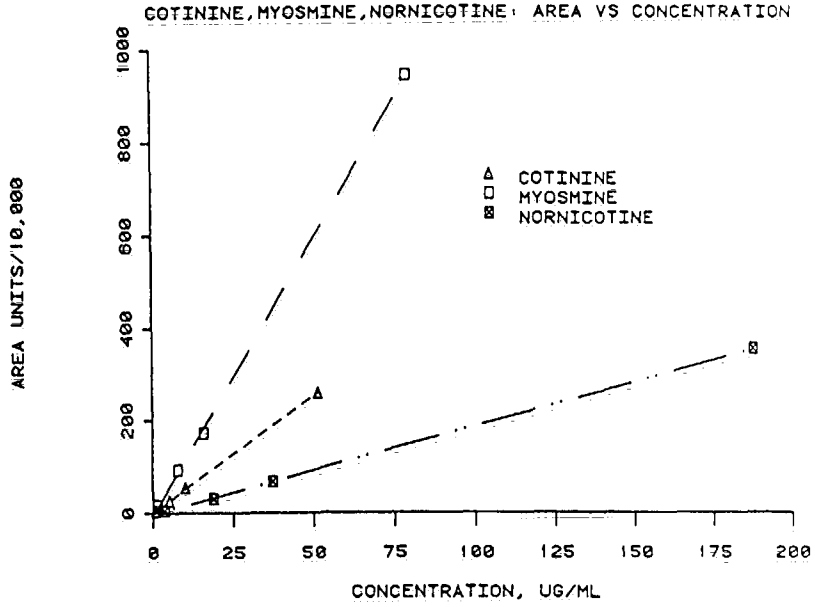


FIGURE 4

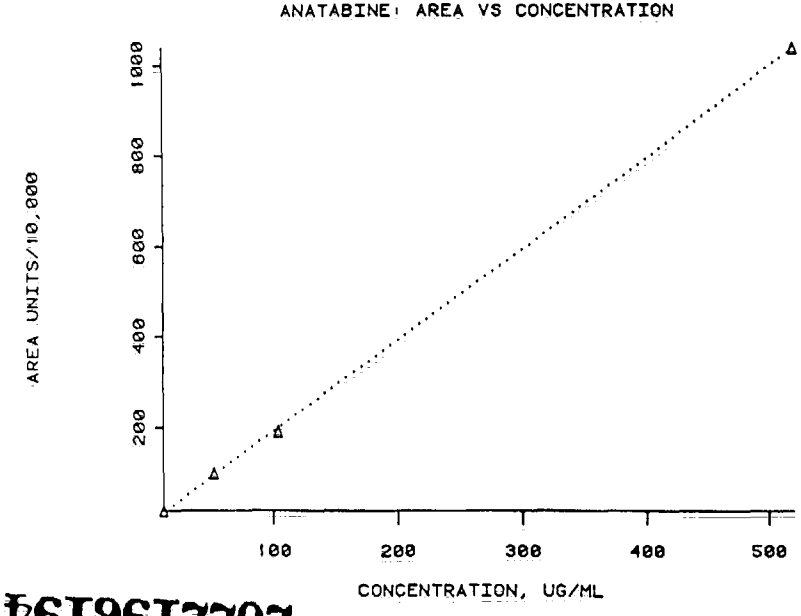
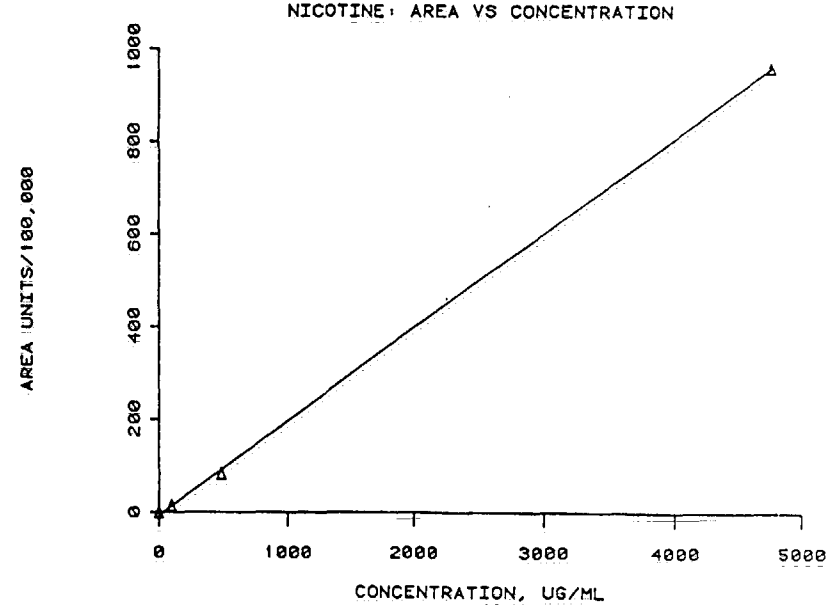


FIGURE 5



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The ion pairs are detected by monitering the effluent at 224 nm. Figure 2 shows a standard curve obtained by plotting peak height vs concentration over a wide range of concentrations. The method is linear over at least the range covered. In practice, accurate results below about 0.001% are not possible due to interferences in tobacco samples. In cases where very low nitrate levels are found, the nitrite levels are below detectable limits.

b. Minor Alkaloid/Nicotine

The method used in the 1984 studies for minor alkaloids was based on a paper by Piade et al.[9], and employed an ODS column with elution by a pH 7.56 triethylamine phosphate buffer with added acetonitrile/THF. There were a number of problems with this method. Columns only lasted for a short time, and new columns sometimes did not work. A new method was developed based on the use of an IBM Phenyl-RP column[10]. Although this method does not allow anatabine and anabasine to be separated, it does allow all the most important alkaloids to be quantitated, and the conditions chosen have resulted in longer column life as well as less difficulty in establishing conditions for new columns. In addition, since nicotine is the last compound to elute, the method allows measurement of minor alkaloids and nicotine in the same run.

The minor alkaloid/nicotine method involves shaking a mixture of 1 g of ground tobacco with aqueous and solid barium hydroxide and a benzene/chloroform solvent, filtering the organic layer, and removing the solvent. The residue is dissolved in the HPLC solvent, which consists of 20% acetonitrile/THF, 200:1, and 80% 0.007M triethylamine phosphate, adjusted to pH 7.1 after mixing. After filtering, the sample is placed in an autosampler vial. The effluent is monitered using 254 nm UV. The

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system includes a computing integrator, and is essentially the same as that used for the nitrate/nitrite method. The standard includes cotinine, myosmine, anatabine, nornicotine, and nicotine. Synthetic nicotine is used to avoid contamination with other alkaloids present in commercially available nicotine. An external standard method is used, since attempts to find a suitable internal standard were not successful. The integrator calculates the alkaloid values as % of the weight of tobacco, and these values are then normally adjusted to dry weight basis. Figures 3 to 5 show the calibration curves for the alkaloids included in the standard solution. Linear response was obtained in each case over the range expected in tobacco samples.

c. Nitrate Reductase Method

A method for tobacco nitrate reductase activity (NRA) was developed in order to allow detection of the enzyme during curing. The procedure described by Garrett for Neurospora crassa[11] was combined with literature methodology available for Nicotiana tabacum[12], and further altered to achieve the desired results. The method has been described in a memo[13]. Basically, the tobacco tissue is homogenized in a buffer solution at a low temperature and centrifuged. Conversion of added nitrate to nitrite in the presence of NADH is measured spectrophotometrically after adding color developing agents. Field grown tobacco had higher levels of NRA than greenhouse grown tobacco when assayed under these conditions.

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2. Nitrosation Studies

a. Reactions of Tobacco Alkaloids with Nitrite

To develop a better understanding of the effects of possible variables on formation of the TSNA, studies have been carried out in which the alkaloids were nitrosated under aqueous conditions. In the previous Annual Report[2], relative yields of the various TSNA from nicotine, nornicotine and anatabine at pH 5.5 were determined. The effect of varying pH between 4.5 and 5.5 on the yields of NNN, NAT, NNA and NNK from commercial nicotine was also determined. This year the studies were expanded to include the effect of varying the pH from 4.0 to 7.5. Both commercial S-nicotine, and synthetic R,S-nicotine[14] were subjected to this treatment to determine whether the NAT observed in earlier studies was formed from anatabine present as an impurity in commercial nicotine or from nicotine itself[15].

The reactions were carried out by allowing nicotine and nitrite (mole ratio 1:1.57) to react at room temperature (22-23°C) for 10 days in dark amber vials in citrate-phosphate buffer solutions adjusted to different pH. After the reaction, the pH was adjusted to 5.0 and the TSNA extracted with methylene chloride. TSNA were measured using GC/TEA, and results were corrected for the recovery of ^{14}C-NNN. Table 1 shows the results obtained from the reactions of both sources of nicotine. The results are also plotted in Figures 6 and 7.

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TABLE 1.
% YIELDS OF TSNA FROM NITROSATION OF SYNTHETIC AND COMMERCIAL NICOTINE AT DIFFERENT pH.
Mole Ratio of Nicotine to Nitrite = 1:1.57;
Reaction Temperature = 22-23°C;
Reaction Time = 10 Days.

	SYNTHETIC (% Yield)			COMMERCIAL (% Yield)			
pH	NNN	NNA	NNK	NNN	NNA	NNK	NAT*
4.0	0.080	0.549	0.087	0.166	0.974	0.120	75.7
4.5	0.075	0.392	0.060	0.134	0.623	0.089	65.9
5.0	0.030	0.233	0.034	0.056	0.410	0.051	54.3
5.5	0.012	0.259	0.033	0.030	0.467	0.086	17.6
6.0	0.005	0.271	0.021	0.008	0.405	0.033	4.7
6.5	0.002	0.147	0.008	0.004	0.290	0.015	2.2
7.0	ND	0.065	0.003	0.003	0.132	0.006	1.9
7.5	ND	0.014	ND	ND	0.025	ND	1.6

* Calculated from anatabine measured in commercial nicotine = 0.284%
ND = not detected

TABLE 2.
MINOR ALKALOIDS FOUND AFTER NITROSATION OF SYNTHETIC NICOTINE OVER A RANGE OF pH
(mg recovered from 10 mg nicotine)

pH	COTININE	MYOSMINE	ANABASINE	ANATABINE	NORNICOTINE
4.0	0.022	0.022	ND	0.016	0.040
4.5	trace	0.011	ND	0.015	0.082
5.0	ND	0.008	ND	0.008	0.037
5.5	0.025	0.021	ND	ND	0.082
6.0	ND	0.015	*	0.031	0.075
6.5	ND	ND	*	ND	ND
7.0	ND	0.004	*	ND	ND
7.5	0.014	0.002	*	ND	ND
UNREACTED		0.001[a]	0.001[b]	*	ND

* Interfering peak
ND = not detected
[a]0.008% [b]0.014%

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FIGURE 6.
NITROSATION OF SYNTHETIC NICOTINE

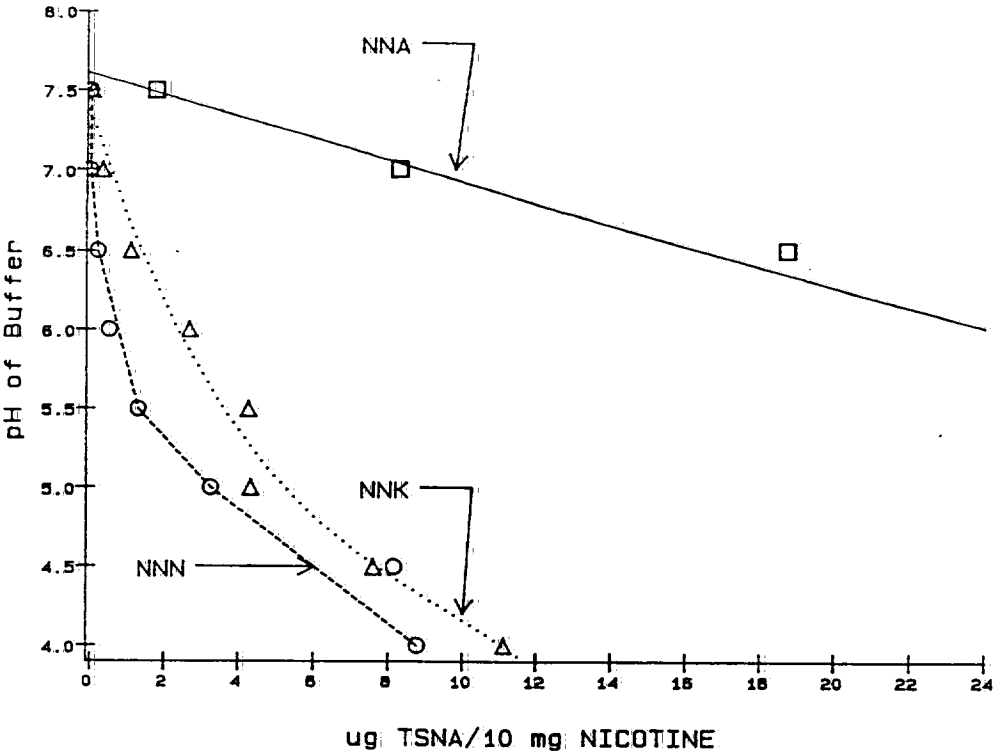
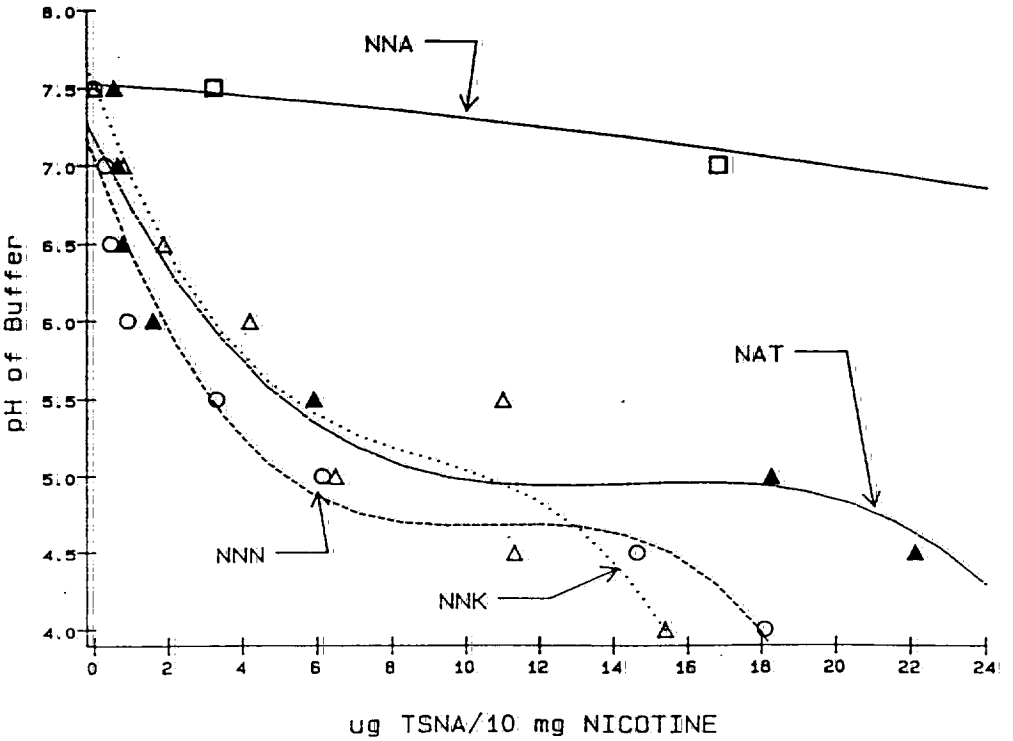


FIGURE 7.
NITROSATION OF COMMERCIAL NICOTINE



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The major difference between the results obtained by starting with synthetic and commercial nicotine was the presence of NAT in the reaction mixture from commercial nicotine. When synthetic nicotine was analyzed by HPLC for the presence of minor alkaloids (Table 2), nornicotine and anatabine were not detected, but 0.284% anatabine was detected in redistilled commercial nicotine. Therefore, the formation of NAT from commercial nicotine is certainly due to the much faster reaction with the anatabine present. This finding indicates that the NAT found in tobacco is due to the simple nitrosation of anatabine, and not to formation from nicotine by oxidative N-nitrosation via NNA as proposed by Tso et al[16].

Minor alkaloids present after the reactions of synthetic nicotine were measured by HPLC. These data are also shown in Table 2. The levels were generally higher after the reactions than those found in synthetic nicotine, and were highest at low pH, indicating a relationship between the formation of these alkaloids and extent of nitrosation. Nornicotine was formed in the largest quantity, possibly indicating that nornicotine is produced as an intermediate during the nitrosation reaction. The mechanism proposed by Hecht[1] to explain the products of nicotine nitrosation includes the formation of nornicotine by hydrolysis of the iminium ion generated by the initial nitrosation of nicotine at relatively low pH (Figure 8). Nornicotine is then nitrosated directly by nitrous acid present in the reaction mixture. The detection of a small amount of a peak having the same retention time as anatabine must be regarded as questionable, since no NAT was detected. The much faster rate of formation of NAT should have resulted in this compound in detectable amounts if any anatabine was present. No attempts have been made to look for the secondary amines corresponding to NNA and NNK,

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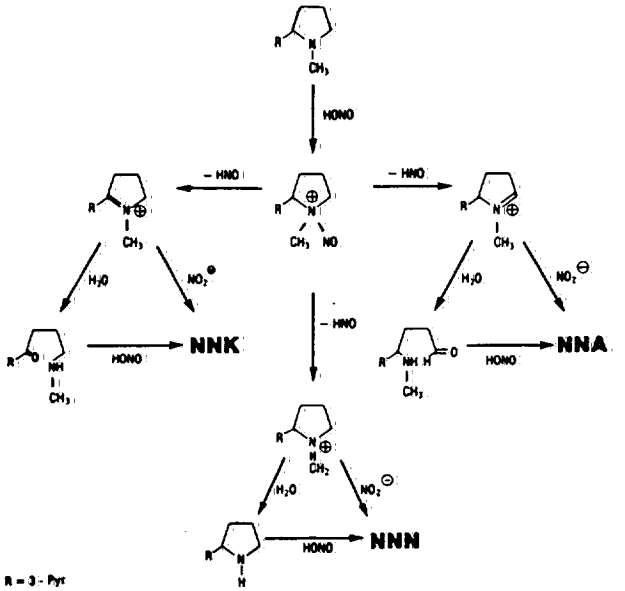
although these were probably present at the lower pH levels, and may actually be present in tobacco.

FIGURE 8.
PROPOSED MECHANISMS FOR NITROSATION OF NICOTINE



All of the TSNA detected were present at higher levels at pH 4 and decreased rapidly after around pH 5. Ratios of individual TSNA also change markedly as a function of pH. Ratios of yields of the individual TSNA to each other from both sources of nicotine are shown in Table 3. Even though there are variations in absolute yields from the two sets of data, the ratios of products are fairly similar. Especially interesting is the large change in NNK:NNN and NNA:NNN between pH 5 and 6. The pH of tobacco is generally in this range. Small variations in pH, therefore, may be expected to have a significant effect on both amounts and ratios of individual TSNA. This may partially explain the difficulty in attempts to correlate TSNA levels in tobacco with precursor levels, since local variations in pH are not uncommon. The effect of pH on ratios may also partially explain the larger NNK/NNN ratio normally found in bright tobacco relative to burley[17].

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TABLE 3.

RATIOS OF YIELDS OF NNA AND NNK TO NNN
FROM NITROSATION OF SYNTHETIC AND COMMERCIAL NICOTINE
(Data from Table 1)

	SYNTHETIC		COMMERCIAL	
pH	NNA:NNN	NNK:NNN	NNA:NNN	NNK:NNN
4.0	6.8	1.1	5.9	0.7
4.5	5.3	0.8	4.7	0.7
5.0	7.8	1.1	7.3	0.9
5.5	22.0	2.8	15.5	2.9
6.0	56.9	4.4	46.7	4.0
6.5	80.3	4.3	73.6	3.7
7.0	*	*	49.6	2.4
7.5	*	*	*	*

* One or both compounds not detected

Time course studies of the nitrosation of nornicotine and synthetic nicotine at pH 5.5 and 22°C were conducted over a period of 10 days, reaction conditions being otherwise similar to those used for the pH studies. Samples were taken at intervals and quenched by adding them to solutions of sodium ascorbate, followed by workup and analysis for TSNA. The data are shown in Tables 4 and 5.

Although there was considerable scatter in the data, presumably due to errors in measuring the TSNA in these samples with such low levels, the number of data points still allow the general time course of the reaction to be followed. As shown in Figures 9 and 10, the yield of NNN from nornicotine increased with time in a fairly linear fashion after the first few hours. The initial rate was much faster, as seen in Figure 9.

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TABLE 4.
NORNICOTINE NITROSATION

Mole Ratio of Nornicotine:
Nitrite = 1:1.42
Temperature = 22°C, pH = 5.5

TIME (Hr)	NNN (%YIELD)
0.0	0.0000
0.5	0.7439
1.0	0.9015
1.5	0.9314
2.0	1.2390
3.0	0.9933
4.0	0.8177
5.0	1.0350
6.0	1.3920
7.0	0.8517
8.0	1.3140
24.0	1.5980
48.0	3.0420
76.5	3.2970
96.0	4.2980
168.0	7.0750
192.0	6.8930
216.0	8.0880
240.0	8.7300

TABLE 5.
R,S-NICOTINE NITROSATION

Mole Ratio of Nicotine:
Nitrite = 1:1.67
Temperature = 22°C, pH = 5.5

TIME (Hr)	% YIELD		
	NNA	NNN	NNK
1	0.002	0.008	0.028
2	0.002	0.007	0.025
3	0.002	0.007	0.025
4	0.005	0.011	0.038
5	0.009	0.019	0.065
6	0.077	0.011	0.036
21	0.037	0.008	0.024
24	0.047	0.008	0.024
48	0.125	0.015	0.055
72	0.189	0.017	0.052
144	0.415	0.039	0.098
168	0.417	0.039	0.082
196	0.460	0.062	0.135
218	0.587	0.080	0.179
239	0.676	0.105	0.255

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FIGURE 9. NORNICOTINE NITROSATION.
TIME COURSE (pH 5.5)
(Data from 1st 10 Hr)

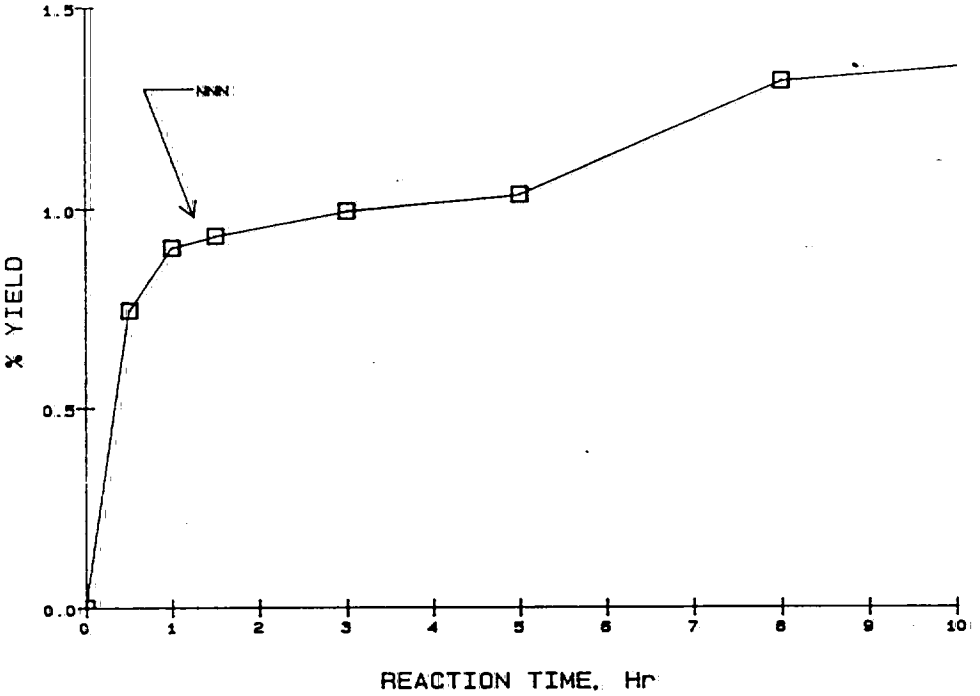

FIGURE 10. NORNICOTINE NITROSATION.
TIME COURSE (pH 5.5)
(Linear Regression)

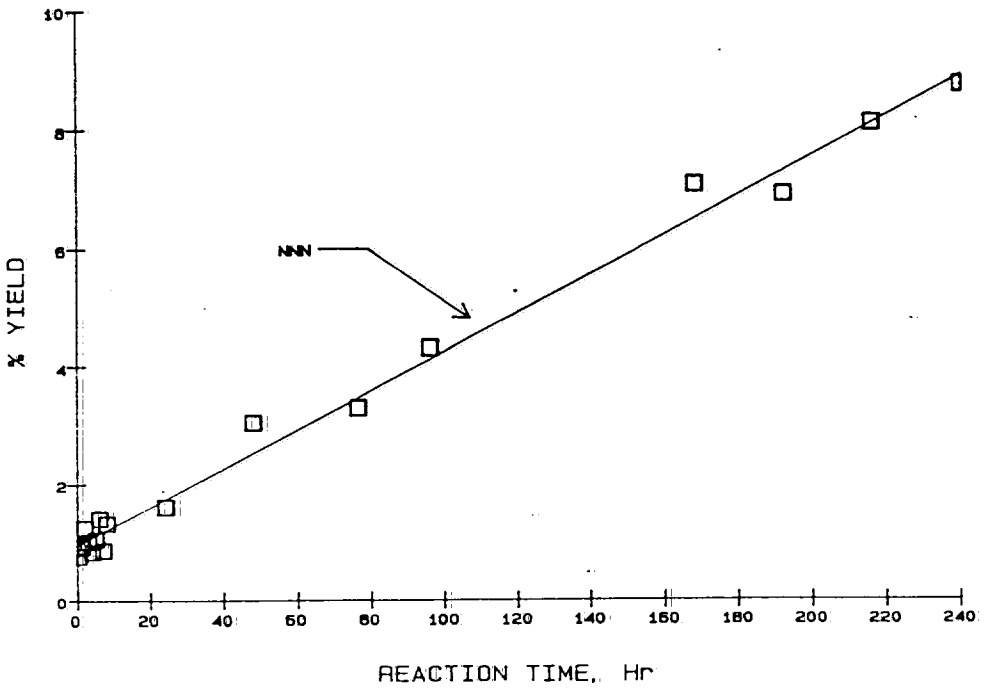

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FIGURE 11. NICOTINE NITROSATION, TIME COURSE
(Data from 1st 24 Hr.)

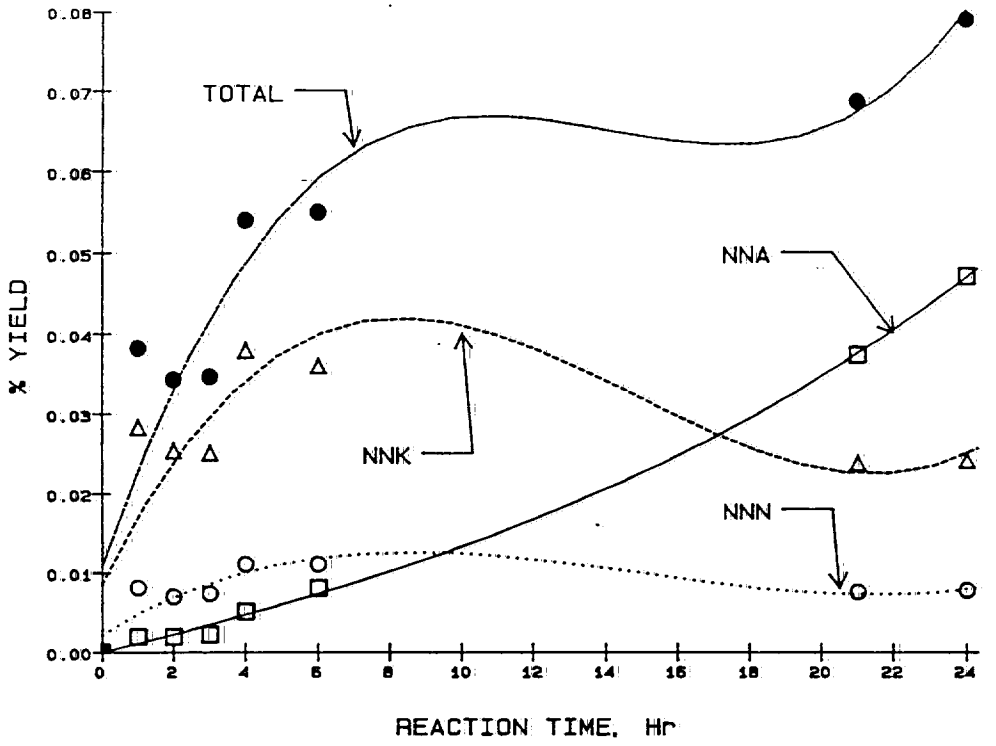
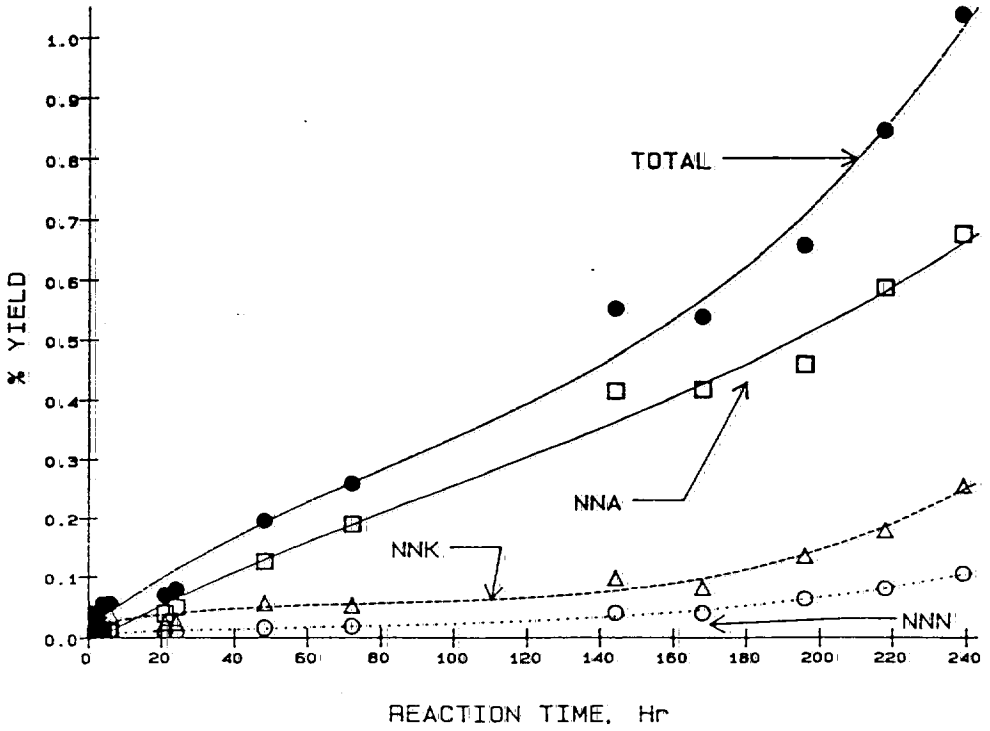


FIGURE 12. NICOTINE NITROSATION,
TIME COURSE



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In Figure 11, the curves for the first 24 Hr of the nicotine nitrosation are shown. Polynomial curves were fitted to the data. The NNN and NNK levels tended to increase to a peak around 8-10 Hr, then decline before increasing again around 20 Hr. Further increases continued for the rest of the reaction time, as shown in Figure 12. However, the NNA concentration increased in a much more regular fashion, indicating that the initial reation rate did not change. The summation of the NNN, NNK and NNA data produced the TOTAL % curves also shown in Figures 11 and 12. This curve showed no decrease in concentration between 8 and 20 hours due to the cancelling of the negative NNN and NNK curves by the NNA curve, possibly indicating some sort of equilibrium between these products.

It appears from these data that there is a difference in the mechanism of formation of NNA compared to NNN and NNK, although all three of these compounds are proposed to result from the corresponding iminium ions (Figure 8). The formation of the iminium ion leading to NNA would be expected to be kinetically favored, explaining the preponderance of NNA. The secondary amine nitrogen atoms of the three intermediates formed by hydrolysis of the iminium ions would be expected to have different pKa's. The pH of the reaction mixture could influence the relative stabilities of these intermediates, and also change the extent of the reverse reactions during formation of the iminium ions. Higher pH increases the formation of NNA relative to NNN and NNK (Table 3). At near neutral pH, the iminium ions can be directly nitrosated, and the product ratios more directly reflect the extent of formation of the iminium ions.

The identification of the peak referred to as NNA here was made by

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inference from the work of Adams[18], since no standard was available. The relative yields of the individual TSNA under similar reaction conditions were of the same order, and relative retention times are similar to those published. There is little doubt that the assignment is correct. However, an effort to confirm this assignment by either GC/MS or comparison of retention data to those of the authentic compound will be made at a future date. Dr. G. Neurath is currently preparing this compound.

Further speculation as to the meaning of the aqueous nitrosation data has not been attempted, because in the actual curing situation the nitrite ion is believed to be generated and consumed during the time course of the reaction rather than being fixed at the initiation of the reaction as was the case in these experiments. Also, competing reactions probably consume more nitrite than nitrosation of the alkaloids, such as nitrosation of phenols or primary amines, or reduction by nitrite reductase.

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b. Organic Nitrosating Agents

The possibility of formation of organic NSA from nitrite and certain organic compounds was presented by Mirvish[19]. Lipid-soluble NSA were shown to be formed when mice were exposed to NO_2. The organic-soluble fraction reacted with dimethylmorpholine to give the corresponding nitrosamine. It was demonstrated that a similar nitrosating agent could be prepared by allowing NO_2 to react with methyl linoleate. At a meeting in 1985[20], Mirvish reported the fractionation of a similar preparation by HPLC, followed by isolation and identification of the NSA as 3-β-cholesteryl nitrite. This compound was also prepared directly by reaction of cholesterol with NO_2.

At the same meeting[20], Hotchkiss showed that NSA were formed by reaction of oxides of nitrogen with fatty acid esters present in bacon fat. These compounds were found to be addition products of the double bonds: C-nitro, C-nitroso, and nitrite ester groups in all possible combinations. Reaction of these NSA with amines took place between 100 and 200°C, with maximum yield of nitrosamines at 170°C.

The information from these studies suggested that a similar mechanism may at least partially explain the formation of TSNA in tobacco during curing. The tobacco terpenes, as well as the sterols and lipids, are potential precursors of organic NSA. A number of alcohols and unsaturated compounds are present in tobacco. There are several possible ways in which this type of reaction could be involved. The organic NSA could be formed before harvest of the tobacco by reaction of nitrite with solanesol[21] or other terpenols present in the chloroplasts. No reaction would be expected to occur between the NSA and the amines at this time due to compartmentalization, an assumption which is supported by the lack

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of TSNA found in green tobacco. However, the NSA could react with the amines during curing, when dehydration with accompanying membrane rupture allows mixing of the NSA with the amines. The formation of the NSA could also occur by a similar process at some time after harvest, from nitrite generated by low levels of NRA, or by bacteria, with nitrosation of the amines following at a later time during curing.

An initial experiment was carried out to determine whether any organic NSA could be detected in tobacco extracts[22]. Methylene chloride extracts of bright tobacco were treated with 2,6-dimethylpiperidine (DMPip) at room temperature as well as under reflux (42°C) conditions. GC/TEA analysis gave no evidence of the formation of nitroso-2,6-dimethylpiperidine (NDMPip). However, the methodology used for this experiment included an evaporation step, and the nitrosamine may have been lost.

To establish the validity of the organic NSA hypothesis for tobacco, chloroform extracts of green bright tobacco and partially cured burley tobacco were prepared from some of the tobacco used for the 1985 curing studies[23]. Ethyl acetate solutions of 500 mg of resin were added to aqueous solutions containing 425 mg of KNO_2 in pH 5.5 citrate/phosphate buffer. The solutions were either stirred for 24 hours at room temperature or refluxed for 10 hours. The organic and aqueous materials were separated by chloroform/brine extraction. The nitrosated bright and burley resins, were then mixed with 2-ethylpiperidine, 120 mg resin and 1 mg base for each sample, in ethyl acetate solution. Samples of the nitrosated resins alone were treated in the same way. These solutions were placed in amber vials and allowed to stand at 37.5°C for 10 days.

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The data from the experiment are shown in Table 6. It was necessary to chromatograph the reaction mixture on alumina/hexane to remove the waxes. The methylene chloride eluates from alumina were analyzed for N-nitroso-2-ethylpiperidine (N-2-EtPip) by GC/TEA. All the samples contained the nitrosamine. However, the two samples where the extracts were nitrosated at room temperature contained the same amount of the nitrosamine, and 4-8 times as much as when the nitrosation was carried out at the higher temperature. The experiment confirms the hypothesis that nitrite can be bound to organic molecules contained in tobacco, and the bound nitrite can be transferred to amines. It appears from the current data, that at least the initial transfer of the -NO function occurs better at room temperature than at higher temperatures.

Plans have been formulated to investigate the organic NSA hypothesis in much greater detail in 1986. These plans are included in the appropriate section of this report.

TABLE 6.

GC/TEA RESPONSE FROM REACTION OF NITROSATED TOBACCO RESINS (120 mg) WITH 2-ETHYLPIPERIDINE (1 mg) IN ETHYL ACETATE SOLUTION. Reaction Time = 10 Days; Reaction Temperature = 37.5°C; pH=5.5

TOBACCO TYPE	RESIN NITROSATION CONDITIONS TEMP., °C	TIME, Hr.	Peak Ht.* N-2-EtPip
BRIGHT	23	24	48554
BRIGHT	80	10	13764
BURLEY	23	24	48367
BURLEY	80	10	5789

* GC/TEA peak corresponding to standard N-2-EtPip

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3. Role of Microorganisms in TSNA Formation

That bacteria are present on the surface of tobacco leaves is no surprise. Some of these microorganisms are capable of reducing nitrate to nitrite. It is doubtful that bacteria have any effect on green tobacco unless there is an injury to the leaf tissue[24]. The pertinent question is whether or not these bacteria exert an effect on TSNA production during curing, aging and/or processing of tobacco.

There have been a number of studies in the literature where bacteria have been implicated in TSNA formation[3,25], but there is no positive evidence that they are actually responsible. Bacterial levels on the leaf surface have been found to be relatively constant during curing[2]. However, bacteria are still considered to be potential sources of NR, and may even be capable of catalyzing TSNA formation[26].

a. Isolates From Green Leaf

As part of the model system studies carried out in 1984[2] several species of bacteria were isolated from green field-grown bright tobacco. These were tested for their abilities to grow, reduce nitrate to nitrite, and produce TSNA when inoculated into sterile filtrates made from homogenates of the same tobacco. The results were reported previously[2]. One of the cultures converted nitrate to nitrite efficiently, and high levels of TSNA were found, but the organism did not exhibit very much growth in the filtrate. Another isolate which was subjected to the same conditions grew to a much greater extent but produced only a fraction of the TSNA. Therefore, it was concluded that it may not be necessary for bacteria to exhibit extensive growth in order for NR to be active and TSNA to be formed.

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As a continuation of this work, 12 bacterial isolates were prepared this year from green field-grown burley tobacco[27]. Preliminary biochemical tests indicated that most of the isolates were Pseudomonas-like organisms[28]. Some of the cultures appeared to be duplicated, and only 10 cultures were chosen for testing. These were inoculated into sterile filtrates made from green field-grown bright tobacco in the same manner as in the 1984 study. All but three cultures reached stationary growth when incubated for 4-6 days at 37.5°C. Nitrite was present in varying amounts in all but three cultures, but TSNA were detected in only one culture. The data are shown in Table 7.

TABLE 7.

INOCULATION OF BACTERIA INTO BRIGHT TOBACCO FILTRATES.

Filtrates were supplemented with nicotine and nitrate. Stock cultures grown up in nutrient broth for 3 days and inoculated into the sterile filtrates. Initial pH = 6.0. Incubated at 37.5°C. Growth followed by plate counting. Samples analyzed when cell concentration stabilized.

CULTURE No.	NICOTINE (mg/ml)	NO_2^- -N (mg/ml)	NO_3^- -N (mg/ml)	NNN (ug/ml)	NAT (ug/ml)	NNK (ug/ml)	FINAL CELLS/ml
1	a						
2	0.573	1.13	86.60	b	0.026	b	6.6e+07
3	0.616	1.11	87.26	b	0.224	b	8.8e+06
4	0.585	0.94	90.64	b	b	b	2.5e+08
5	a						
6	0.591	b	87.26	b	b	b	4.4e+06
7	a						
10	0.640	b	90.80	b	b	b	1.5e+07
11	0.597	0.75	92.95	b	b	b	1.0e+08
12	0.597	b	97.87	0.169	2.180	0.075	1.0e+09

a No growth; not analyzed.
b Not detected.

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TABLE 8.

INOCULATION OF BACTERIA INTO BURLEY TOBACCO FILTRATES.
Cultures and conditions as described in Table 7, except that no nicotine or nitrate added. All analytical values reported per gram dry weight of tobacco represented.

CULTURE No.	NICOTINE (mg/g)	NO_2^--N (ug/g)	NO_3^--N (ug/g)	NNN (ug/g)	NAT (ug/g)	NNK (ug/g)	FINAL CELLS/ml	FINAL pH
1	33.15	42.9	345.0	0.17	0.98	0.23	100	7.7
3	18.09	34.3	524.0	0.07	1.25	0.08	2.0e+04	7.8
4	30.69	426.2	1400.0	4.78	24.76	0.24	<100	6.0
5	34.71	510.8	1444.0	6.11	29.68	0.24	3.0e+03	5.6
8	40.35	1411.8	28.4	6.99	68.00	0.15	3.3e+04	6.3
10	40.73	a	a	0.10	0.62	0.11	1.0e+04	7.7
12	40.13	a	a	0.12	29.24	0.28	<100	7.7

a Not detected.

The organisms were also inoculated into sterile filtrates prepared from green field-grown burley tobacco[27]. The samples were incubated at 37.5°C for 7 days. The data are shown in Table 8. TSNA were formed in all the samples in varying amounts, although the organisms grew poorly. There was no apparent correlation between nitrate, nitrite and TSNA levels in the data generated[29].

Alcaligenes denitrificans is capable of depleting nitrite via NR. This should facilitate a decrease in TSNA levels under appropriate conditions. An experiment was carried out to evaluate this possibility in the sterile filtrate model[30]. Sterile filtrates from bright and burley tobacco were used. To allow better growth of the organism, the pH was adjusted to 6.0 before inoculation. Also, the bright filtrate was supplemented with nicotine and nitrate due to low levels present in the initial filtrate. The cultures were incubated under anaerobic conditions for 7 days at 37.5°C. The organism grew well in both filtrates, and attained final pH values of 7.2 and 7.6, respectively, for burley and bright. The data are shown in Table 9. No TSNA were detected in the

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bright filtrates. In the burley filtrates, the levels of TSNA were half the levels found in a control that was not inoculated, and the nitrite level in the inoculated sample was also less than that of the control[29].

TABLE 9.

INOCULATION OF A. DENITRIFICANS INTO BURLEY AND BRIGHT FILTRATES. Incubated at 37.5°C. Initial pH = 6.0. Values reported per gram dry weight of tobacco represented. Bright filtrate suplemented with nicotine and nitrate.

SAMPLE	NICOTINE (mg/g)	NO_2^--N (ug/g)	NO_3^--N (ug/g)	NNN (ug/g)	NAT (ug/g)	NNK (ug/g)	FINAL CELLS/g	FINAL pH
Burley control	32.46	83.3	2057	0.330	1.667	0.101	b	5.8
Burley + Cells	34.89	66.7	a	0.192	0.970	0.050	3.45e+09	7.2
Bright Control	26.21	a	3647	a	0.039	a	b	5.8
Bright + Cells	18.70	a	a	0.016	0.042	0.040	5.28e+10	7.6

a Not detected. b No growth (not inoculated).

The results of the above experiment indicate that A. denitrificans is capable of carrying out the desired removal of nitrite if the pH is high enough for the organism to grow. However, at pH levels this high, little nitrosation is likely to occur in an aqueous system.

b. Sterile Tobacco

One method of determining the contribution of bacteria to the reduction of nitrate and/or formation of TSNA would be to cure sterile tobacco and subsequently measure nitrate, nitrite and TSNA levels. If technology to carry out this experiment could be developed, and if the curing proceeded otherwise normally, the presence or absence of TSNA relative to a non-sterile control should answer this question. Work done in 1985 on this approach was limited to investigating methodology for sterilization and for curing the sterile leaves under sterile conditions.

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Two types of methods were tried in attempts to sterilize green tobacco. These involved either chemical treatment or use of gamma irradiation. The results were described recently[31], and are summarized here.

For the chemical treatment, three chemical solutions were applied to the detached leaves by soaking for 15 or 30 minutes. The sterilants used were: 95% ethanol, 25% Chlorox, and Incyte (a commercial plant tissue sterilant). The leaves were checked for sterility. Chlorox and Incyte produced more sterile samples than did ethanol, but sterility was not reproducible. Soaking produced visible changes in the leaf surface. A larger scale experiment was carried out in which 40 burley leaves (Ky 10, greenhouse grown) were sprayed on both sides with distilled water, 25% Chlorox, or Incyte, and the solutions allowed to remain 30 minutes followed by washing with sterile water and drying. These, as well as an unsprayed control, were cured in sterile plastic bags in a laminar flow hood. After 8 weeks, the Incyte treated samples were contaminated. The Chlorox-treated leaves were contaminated after 3 weeks. In some cases the water-treated samples remained sterile. A further attempt was made to use Incyte followed by flue-curing. Again, cultures taken during curing showed contamination of the leaves.

Two attempts were made to sterilize green tobacco leaves with ^{60}Co gamma-irradiation. The treatments were done at Georgia Institute of Technology in cooperation with R. Jenkins and R. Newman. In the first experiment, doses of 0.5 to 6 Mrad were employed. At 2.5 Mrad and above, radiation damage to the leaves occurred. As the dose increased, nitrate decreased and nitrite increased. TSNA levels paralleled the nitrite levels. The samples were all sterile, but TSNA were present in all

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samples as well. In the second attempt, doses ranged from 0.001 to 0.5 Mrad. None of these samples were sterile, although there was no reduction of nitrate to nitrite.

The lack of methodology for obtaining sterile tobacco has thus far prevented reasonable progress toward answering the question of bacterial contribution to TSNA formation during curing. Further attempts are planned in 1986, including the use of ethylene oxide in sealed chambers which would allow more complete control of the environment to which the leaves are subjected.

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4. Curing Studies

a. Methodology for Analytical Samples

Based on the previous experience obtained in carrying out curing studies, methodology was devised to reduce scatter in the data due to variations in sampling techniques, etc., and to accommodate the larger scale studies that were planned for 1985. The method used for sample handling before analysis is described here in brief form. The protocol used for leaf dissection was arrived at by consideration of results that were obtained in experiments described under the V446 curing study.

In the case of bright tobacco, the leaves were primed. For burley and V446, the plants were stalk-cut. Except for this, the samples were all handled the same way. Zero-time samples were all primed leaves. The whole leaves were placed in a styrafoam cooler and layered with dry ice to allow rapid freezing. The frozen leaves were broken up, and the midvein and secondary veins removed before freeze-drying. For samples taken later during curing, the middle 3 to 4 sections of each leaf were cut out starting at the midvein and proceeding to the edge of the leaf, along the borders outlined by but not including the veins. The sections of leaf belonging to a given sampling were combined, frozen in liquid nitrogen, and then freeze-dried. Samples were ground in a Wiley mill to pass a 40 mesh screen, desiccated over Drierite overnight, and stored in screw-capped vials in a freezer until analyzed.

OV measurements were made on the ground, desiccated tobacco by standard methods (100°C oven, 3 hr). Duplicate 1 g samples were used for minor alkaloids and for nitrate/nitrite analysis by the methods described in this report. Duplicate 2.5 g samples were analyzed for TSNA by the standard filler analysis procedure used in Project 6908[32]. In the case

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of the burley study, samples were also sent to Analytical Research (AR) for calcium, nitrate, and total alkaloid analysis. Sampling and analysis procedures for bacteria and NRA are described elsewhere in this report.

b. V446 Curing Study (Nornicotine Converter Strain)

The objectives of this study were to determine whether conversion of nicotine to nornicotine could be observed in a nornicotine converter strain during air-curing, and whether this would result in a substantial increase in the ratio of NNN to the other TSNA.

In 1983, Chamberlain and Arrendale[6] reported that although flue-cured V446 tobacco contained nornicotine at 10 times a "normal" level, there was no NNN found. This result appeared to be very unusual, since it is a logical extension from the chemical experiments we and others have carried out that a higher nornicotine to nicotine ratio should result in the formation of more NNN.

Several other studies have appeared in the literature recently in which nornicotine converter strains have been included. Andersen and Kasperbauer[25] reported greatly increased nornicotine and NNN in Ky14-322 when cured by HLC, compared to other burley strains. Andersen and Kemp[4] also reported large increases in NNN and NNK in an HLC processed nornicotine converter strain. MacKowen et al.[3] reported that Ky78-379 after air-curing gave NNN:NAT:NNK ratios of 25:4:1 and 38:9:1 compared to 8:4:1 and 10:8:1 for Burley 21. These studies appear to refute the results obtained by Chamberlain and Arrendale, although the latter data were obtained by flue-curing rather than air-curing or HLC.

V446 Tobacco (20 plants) was grown in the R&D greenhouse by Roger Bass et al. (Project 2525), using double the normal amount of fertilizer per day in an attempt to maximize the nitrate level in the plants at

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harvest. The mature plants were stalk-cut on 9/16/85 and allowed to wilt until the next day. Air-curing was carried out over a 9 week period in a humidity and temperature controlled laboratory on wood racks. After being allowed to remain uncovered for 2-3 days to hasten initiation of drying, the plants were covered with black plastic to prevent moisture loss at too rapid a rate, and keep light exposure to a minimum. Sampling was carried out on the low, middle, and upper stalk positions at zero-time, after 1, 2 and 5 weeks of curing, and after the full 9 weeks[33].

The zero-time samples were handled somewhat differently from the samples taken during and after curing. An attempt was made to determine the distribution of alkaloids and nitrate as a function of location in the leaves to serve as a guide for later sampling. The lower and middle-stalk positions were combined for this purpose to increase the amount of material per sample. The upper-stalk leaves were handled separately. Each leaf was dissected and the lamina tissue near the base and center of the leaf was divided into inner and outer portions. The lamina at the tip was kept as a separate sample. The secondary veins (or veinules) were combined; the whole midvein constituted a sample. The samples were freeze-dried and analyzed as described above for nitrate/nitrite and minor alkaloids/nicotine. The data are shown in Tables 10 and 11.

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TABLE 10.
DISTRIBUTION OF NITRATE AND ALKALOIDS
IN MATURE V446 LEAVES (UPPER STALK POSITION)

LEAF AREA	%NO_3^--N	%MYOS	%ANAT	%NORN	%NIC
INNER/BASE	0.0030	0.0017	0.0677	0.2284	2.986
OUTER/BASE	0.0024	0.0012	0.0850	0.2046	3.527
INNER/CENTER	0.0013	0.0018	0.0844	0.3412	3.394
OUTER/CENTER	0.0012	0.0018	0.0703	0.5410	2.800
TIP	0.0013	0.0051	0.0780	0.6514	2.875
MIDVEIN	0.4842	ND	0.0210	0.0325	0.882
VEINULES	0.1488	0.0008	0.0468	0.1337	1.684

TABLE 11.
DISTRIBUTION OF NITRATE AND ALKALOIDS
IN MATURE V446 LEAVES (LOWER-MIDDLE STALK POSITION)

LEAF AREA	%NO_3^--N	%MYOS	%ANAT	%NORN	%NIC
INNER/BASE	0.0073	0.0007	0.0433	0.1612	2.2370
OUTER/BASE		0.0027	0.0278	0.3759	2.0730
INNER/CENTER	0.0014	0.0080	0.0355	0.3372	2.3690
OUTER/CENTER	0.0013	0.0050	ND	0.8107	1.6720
TIP	0.0013	0.0064	ND	0.9071	0.8103
MIDVEIN	0.2985	ND	0.0151	0.0301	0.7330
VEINULES	0.0026	0.0023	0.0184	0.1939	1.1530

Nitrite was not detected in these samples. Nitrate was concentrated in the midveins and veinules. Concentrations of alkaloids were much lower in these two structures than in the lamina. Nornicotine levels were highest in tip and outer/center sections, and nicotine was highest in the inner/center and base. The decision was made to sample the center section, including inner and outer portions, for the remainder of the study, since the variations in the data for these sections were minimal.

The OV of the tobacco decreased gradually from 85 to 65% during the first 5 weeks of curing, then dropped to 15% in the 6th week, after which an average of 14.5% was measured. These measurements were made on the upper-stalk leaves only. No attempt was made to obtain OV measurements at all stalk positions, due to the scarcity of material.

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Table 12 shows the nitrate and alkaloid data for the samples obtained during and after curing. Again, nitrite was not detected in any of the samples. Nitrate levels were considerably lower than normally seen in tobacco grown under burley conditions, and also lower than normal for bright tobacco. However, the data showed a considerable increase in nitrate at each stalk position as curing progressed. The drastic decrease in nicotine and increase in nornicotine due to the presence of the demethylase system in V446 can be seen by comparing the data in Tables 10 and 11 with those in Table 12.

TABLE 12.
NITRATE AND ALKALOIDS IN V446 DURING CURING

WEEK	%NO_3^--N	%COT	%MYOS	%ANAT	%NORN	%NIC
LOW STALK						
1	0.0029	0.0083	0.0326	ND	4.3113	0.1700
2	0.0060	0.0030	0.0181	0.0146	1.8037	0.0239
5	0.0154	0.0024	0.0180	0.0918	2.2081	0.1371
9	0.0128	ND	0.0130	0.0980	1.8000	0.1461
MID STALK						
1	0.0028	0.0066	0.0116	ND	3.5059	3.6948
2	0.0026	0.0065	0.0745	ND	4.7276	0.0201
5	0.0107	0.0106	0.0251	0.0988	3.1715	0.0939
9	0.0148	ND	0.0221	0.1257	2.6881	0.1115
UPPER STALK						
1	0.0078	0.0043	0.0071	0.0218	1.5843	4.8290
2	0.0057	0.0044	0.0469	ND	4.9479	0.3416
5	0.0221	0.0150	0.0339	0.1044	4.6507	0.4156
9	0.0196	ND	0.0274	0.1454	4.1508	0.3574

The alkaloid data obtained during curing of V446 are shown in graph form in Figures 13, 14 and 15. The increase in nornicotine at the expense of nicotine occurred largely in the first or second week of curing. The data in Table 12 show a considerable increase in anatabine

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FIGURE 13.
ALKALOIDS IN V446 DURING AIR-CURING
(LOW-STALK)

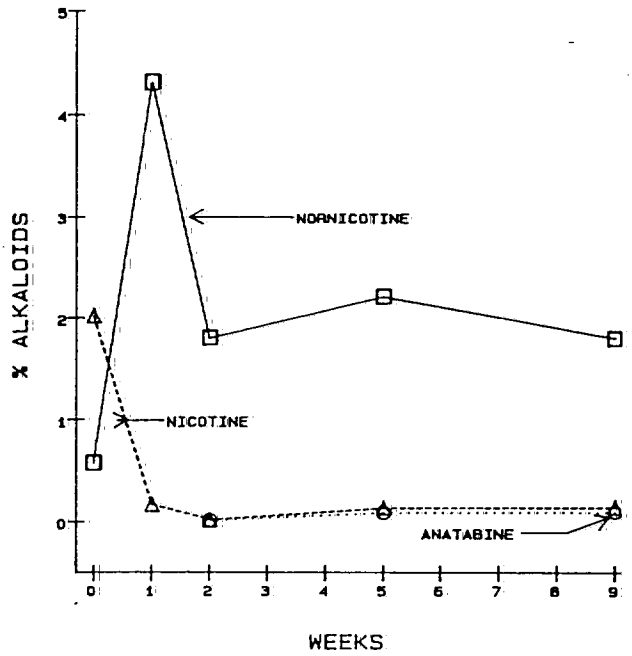


FIGURE 14.
ALKALOIDS IN V446 DURING AIR-CURING
(MID-STALK)

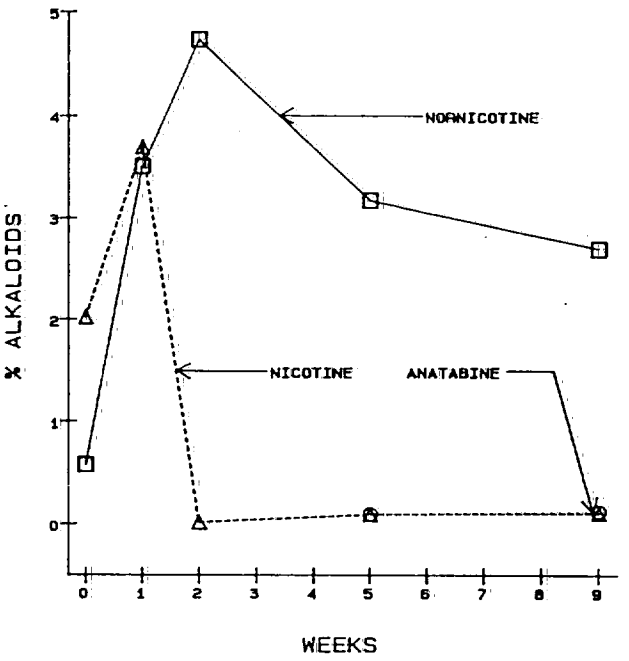
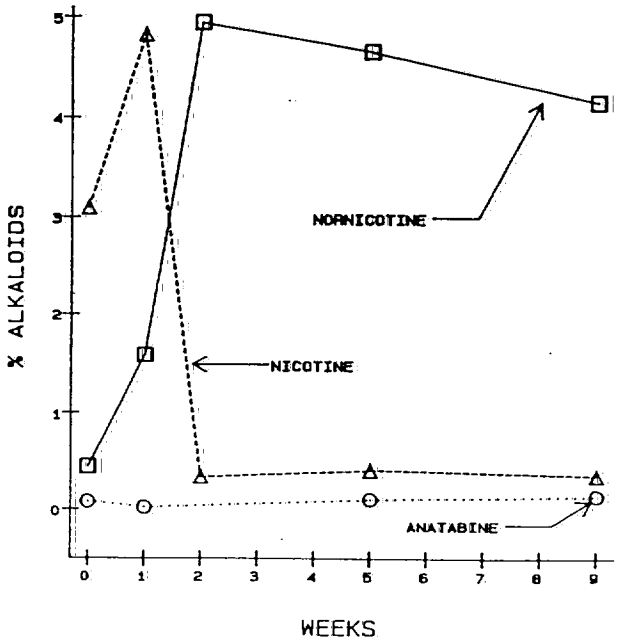


FIGURE 15.
ALKALOIDS IN V446 DURING AIR-CURING
(UPPER-STALK)



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between weeks 2 and 5 as well, although the scale chosen for the graphs does not show this increase.

The TSNA data are shown in Table 13. The data from the low and mid-stalk positions are also plotted in Figures 16 and 17, respectively. NNK levels were very low or not detected. There was a general increase in all the TSNA for the length of the curing time. The larger increase in NNN is easily seen in the graphs. The final ratios of NNN:NAT:NNK for low, mid, and upper-stalk positions were 30:4.4:1, 20:2:1 and 22:3.1:1, respectively. These ratios are similar to those observed by MacKowen et al.[3] for Ky78-379.

TABLE 13.
TSNA IN V446 DURING CURING

WEEK	TSNA, n/g (±SD) NNN	NAT	NNK
LOW STALK			
1	80 ± 5	11 ± 18	ND
2	599 ± 4	59 ± 51	ND
5	1354 ± 11	190 ± 14	21 ± 37
9	1095 ± 15	158 ± 11	36 ± 31
MID STALK			
1	23 ± 22	ND	ND
2	76 ± 1	ND	ND
5	260 ± 13	16 ± 28	ND
9	439 ± 19	43 ± 37	22 ± 39
UPPER STALK			
1	NA	NA	
2	62 ± 7	21 ± 37	ND
5	274 ± 9	38 ± 33	ND
9	479 ± 11	68 ± 1	22 ± 38

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FIGURE 16.
TSNA FORMATION IN V446 TOBACCO
(LOW-STALK)

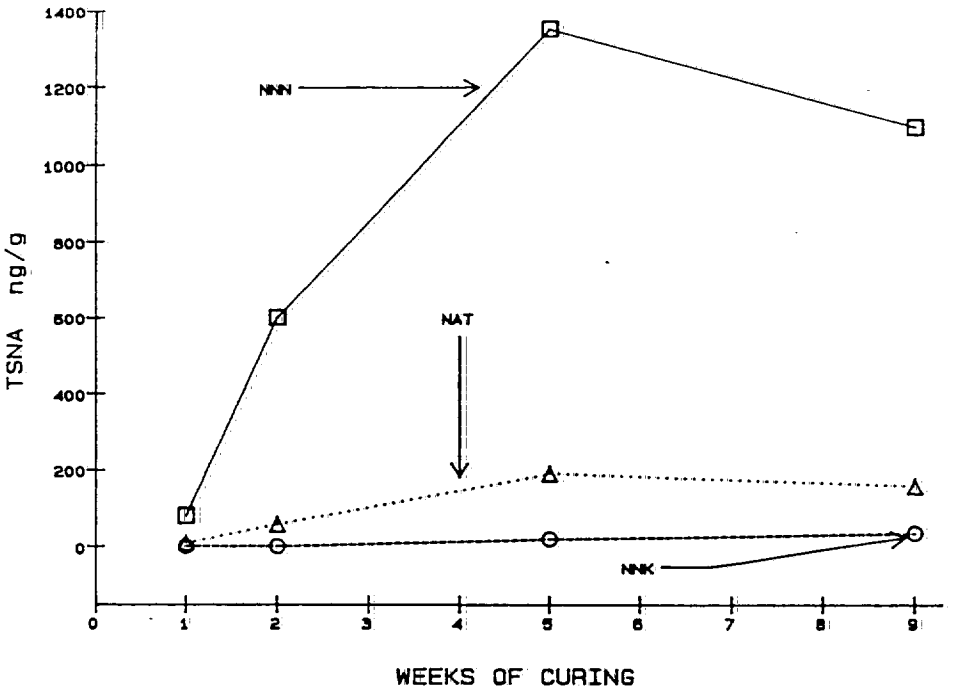
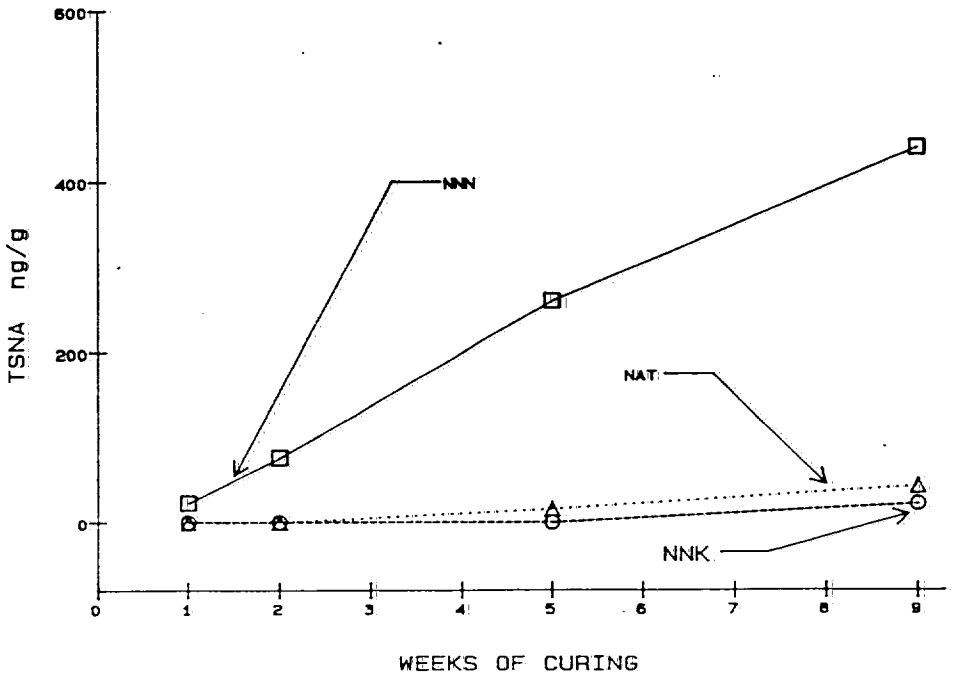


FIGURE 17.
TSNA FORMATION IN V446 TOBACCO
(MID-STALK)



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It can be concluded that in nornicotine converter strains, air-curing leads to high levels of NNN relative to NAT and NNK due to the high ultimate level of nornicotine. Also, it would appear that nornicotine is the predominant precursor of NNN.

The total protein content of the V446 tobacco (determined by the Bio-Rad method) showed a gradual decrease during the first 5 weeks and reached a constant level thereafter[34]. Soluble proteins in the extracts prepared from each sample were separated and analyzed by SDS-Page. Many high MW proteins (>200,000 KD) were found in the first and second week samples, but many of these disappeared thereafter. Thus, it appears that major proteolytic activity took place during the first two weeks of curing. No detectable NRA was present in any of the samples, including the zero-time sample.

c. Bright Tobacco Curing Study

The objectives of this study were: 1. to determine the time-course of chemical, biological, and physical factors which may affect the formation of TSNA in bright tobacco during flue-curing; and 2. to investigate the effects of known nitrosation inhibitors on TSNA formation during flue-curing.

Coker 319 tobacco was field-grown in Appomattox County, Virginia, in conjunction with Dr. Dan Teng (Project 1901). Although the expected date for the first priming was approximately July 15, 1985, heavy rainfall during the month of July caused regreening of the tobacco, and the first priming was delayed until August 14, 1985. Approximately 300 untreated leaves were flue-cured by Dr. Teng in one rack of the curing chamber in

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D-118. The remainder of the leaves were treated with one of four different mixtures:

1. d,l-α-Tocopheryl Acetate 47 g (0.28 g/g DW)
   Tween 80 8 g
   Distilled Water 1 l

2. Ascorbyl Palmitate 41 g (0.24 g/g DW)
   Tween 80 7 g
   Distilled Water 2 l

3. Ascorbic Acid * 17.6 g (0.18 g/g DW)
   d,l-α-Tocopheryl Acetate 47 g (0.28 g/g DW)
   Tween 80 8 g
   Distilled Water 1 l

   * pH adjusted to 5.3 with NaOH

4. Tween 80 8 g
   Distilled Water 1 l

The leaves were sprayed with the above emulsions, allowing the surface to become wet[35]. Experiments where the volume was measured gave the approximate application weights shown per gram of dry weight. The leaves were allowed to dry by hanging in the laboratory before being placed in the curing rack. The treated and untreated leaves were cured at the same time. The untreated leaves were sampled according to the method outlined above at zero-time, after yellowing, when fixed, and when fully cured. All the samples were freeze-dried as described earlier. The samples were analyzed for nitrate/nitrite, minor alkaloids/nicotine, TSNA, NRA and bacteria. The treated leaves were sampled only after full curing, and only the nitrate/nitrite, minor alkaloids/nicotine, and TSNA data were obtained.

In addition, samples of the same tobacco harvested at various times during growth were obtained from Dr. Teng[36]. The data obtained from these samples[37] are shown in Table 14, and are plotted in Figure 18.

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The decrease in nitrate content of the lamina as a function of time is obvious. It is normal for nitrate to decrease after topping because fertilization is usually stopped at this time, but the extreme conditions probably caused a much more drastic depletion than normal. The increase in alkaloids is a normal result of topping.

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

TABLE 14.
ANALYTICAL DATA FROM GREEN BRIGHT TOBACCO
AT DIFFERENT STAGES

WEEKS AFTER TOPPING	%NO_2^--N	%NO_3^--N	%ANAT	%NORN	%NIC
-2	0.0017	0.1678	0.0379	0.0192	0.2653
0	0.0008	0.1050	0.0454	0.0329	1.5708
2	ND	0.0258	0.1215	0.0810	3.8003
4	0.0009	0.0143	0.1194	0.0760	3.0993
6	0.0010	0.0308	0.2224	0.1498	5.2490
7	ND	0.0051	0.1865	0.1308	4.5273
10	ND	0.0010	0.1953	0.1130	4.1747

TABLE 15.
FLUE-CURING OF BRIGHT TOBACCO*

SAMPLE	%NO_3^--N	%ANAT	%NORNIC	%NICOT	TSNA, ng/g ± SD		
					NNN	NAT	NNK
GREEN LAMINA	0.007	0.202	0.096	2.608	60 ± 4	103 ± 10	ND
YELLOWED LAMINA	0.039	0.178	0.048	2.560	63 ± 13	370 ± 89	ND
FIXED LAMINA	0.038	0.229	0.222	3.046	86 ± 13	315 ± 97	27 ± 44
CURED LAMINA	0.002	0.101	0.097	2.576	76 ± 13	245 ± 22	20 ± 32
GREEN STEMS	0.171	0.028	0.013	0.364	60 ± 12	529 ± 65	10 ± 25
CURED STEMS	0.242	0.034	0.007	0.370	51 ± 13	144 ± 61	49 ± 56
CURED VIT E ACET	0.002	0.167	0.164	2.148	79 ± 19	185 ± 16	52 ± 41
CURED VIT C PALM	0.004	0.185	0.126	2.406	79 ± 18	128 ± 15	13 ± 36
CURED VIT C+E ACE	0.001	0.169	0.088	2.238	63 ± 13	178 ± 22	23 ± 34
SPRAYED CONTROL	0.002	0.161	0.077	2.642	65 ± 7	139 ± 11	40 ± 30

*Coker 319, grown in Appomattox, Va., 1985 (dry weight basis).

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

FIGURE 18. CHANGES IN NITRATE AND ALKALOIDS
IN BRIGHT TOBACCO AS A FUNCTION OF TIME VS TOPPING

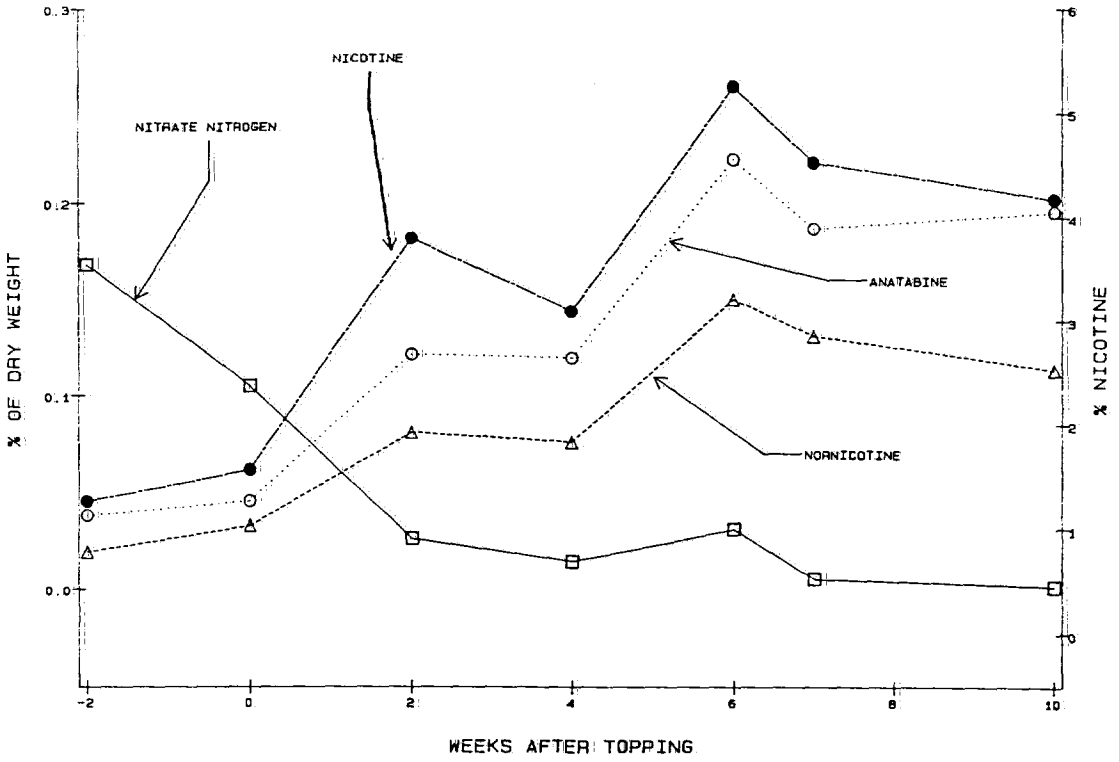


FIGURE 19.
FLUE-CURING OF BRIGHT TOBACCO
(UNTREATED)

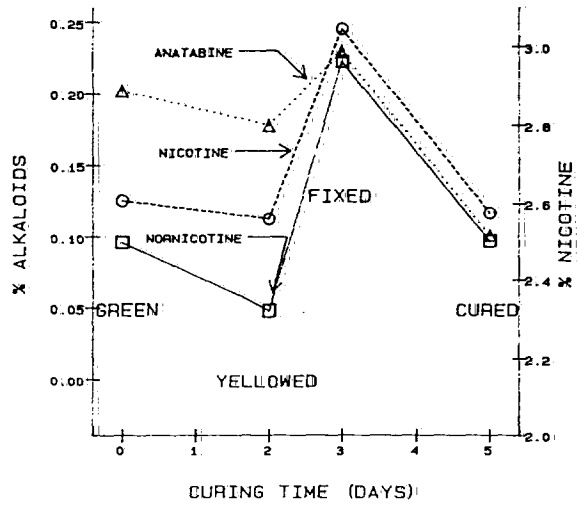
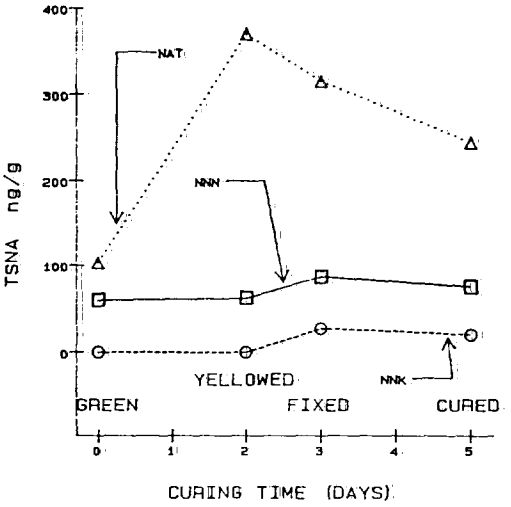


FIGURE 20.
TSNA FORMATION DURING FLUE-CURING
OF BRIGHT TOBACCO



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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

The analytical data obtained during curing[38] are shown in Table 15. As expected, nitrate was concentrated in the stems but very low in the lamina. Nitrite was not detected in any of these samples, and no NRA was found. The alkaloid and TSNA data are plotted in Figures 19 and 20. Due to the extremely low nitrate levels in this tobacco, very low TSNA values were obtained. The data were atypical for bright tobacco in that the NNK values were lower than the NNN values. In other studies where bright tobacco was flue-cured, NNK values were similar to or greater than the NNN values.

The effects of the treatments applied are shown in Figure 21. Although there were some differences between absolute and relative amounts of individual TSNA, there did not appear to be any easily explainable pattern indicating that any of the treatments was responsible for these differences. Rather, the differences appeared to be due to natural variations among samples.

The unusually low nitrate level in this tobacco precluded the attainment of the objectives stated for this study, in that the TSNA levels were loo low and tobacco too different from normal for reliable results to be obtained. The major conclusion that was reached from these results was that very low nitrate levels in bright tobacco lead to extremely low TSNA levels. Data obtained in Project 6908 also show that smoke TSNA levels from cigarettes made from this tobacco were very low as well[39].

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

d. Burley Tobacco Curing Study

The objectives of this study were: 1. to determine the time-course of chemical, biological, and physical factors which may affect the formation of TSNA in burley tobacco during air-curing; and 2. to investigate the effects of known nitrosation inhibitors on TSNA formation during air-curing.

Ky 14 burley tobacco was field-grown in Appomattox County, Virginia, on the same farm as that on which the bright tobacco was grown. This part of Appomattox County is situated on a boundry line where the soil type changes from light, bright-tobacco soil to heavier, burley-type soil. Therefore, both types of tobacco are grown on different parts of the same farm. Approximately 300 plants were stalk-cut on September 17, 1985, speared onto sticks, and allowed to wilt in the field for two days[33]. The sticks were then transported to R&D by truck on a rack. Treatment of 15 plants each with 4 separate emulsions identical to those listed for the bright study was carried out by spraying both sides of each leaf. The treated plants as well as the untreated plants were placed on a large wood rack in a humidity and temperature-controlled lab on T-6. The treated plants were allowed to dry overnight before covering all the plants with black polyethylene sheet plastic. Zero-time sampling was carried out as described earlier on the wilted untreated plants, low, middle and upper stalk positions[40]. The untreated tobacco was sampled once each week until fully cured (9 weeks), and the treated tobacco was sampled after the first and ninth week. Analytical data were obtained on all the samples, but microbiological data were obtained on the untreated samples only. In addition to the analyses obtained for the bright study samples, all of the untreated burley samples were submitted to the AR Division for calcium, total alkaloids and nitrate.

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

FIGURE 21. EFFECT OF TREATMENTS ON TSNA IN CURED BRIGHT TOBACCO

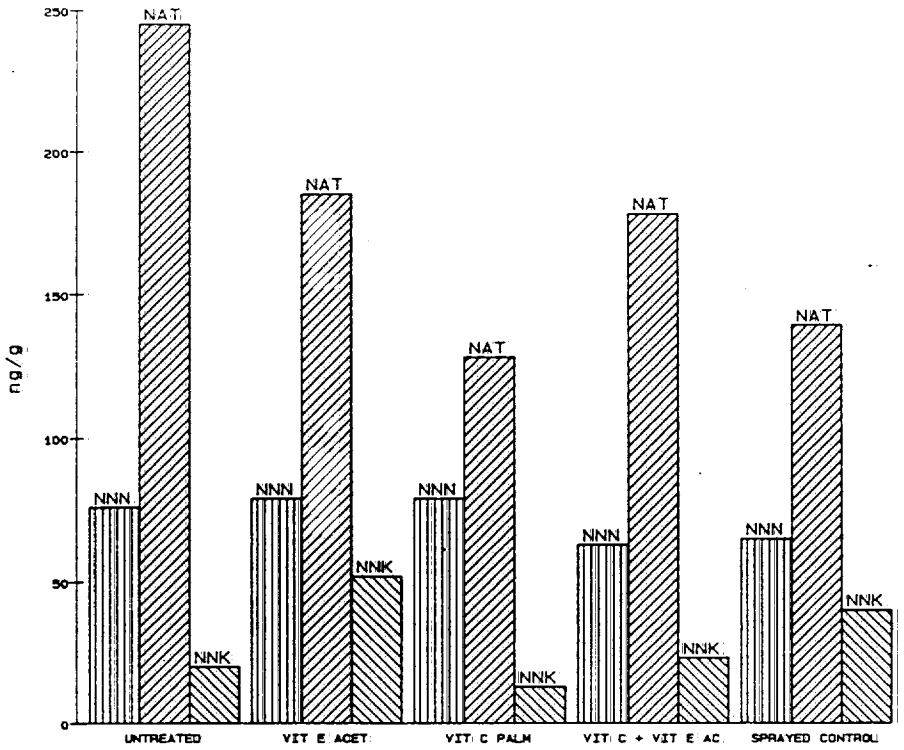
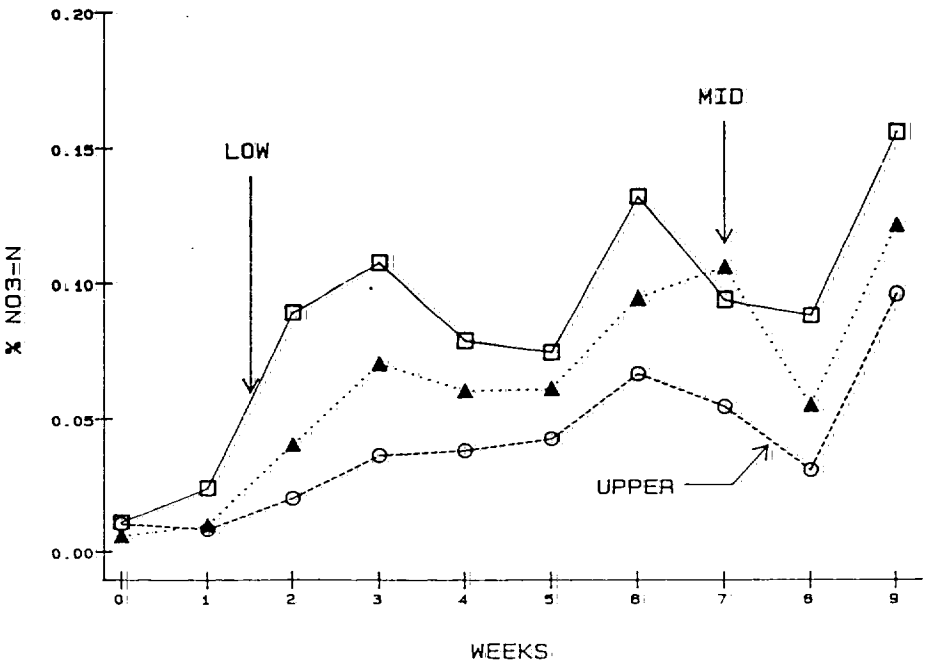


FIGURE 22.
% NITRATE-NITROGEN IN BURLEY
DURING CURING



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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

TABLE 16.
AIR-CURING OF UNTREATED BURLEY TOBACCO (Dry Weight Basis)

WEEKS	$\%NO_3^-$-N[a]	$\%NO_3^-$-N[b]	%Ca	%TA	% ANAT	% NORNIC	% NICOT	TSNA, ng/g (±SD) NNN	NAT	NNK
LOW-STALK										
0	0.011	<.04	2.75	6.41	0.263	0.175	8.01	23 ± 3	100 ± 6	16 ± 26
1	0.024	<.04	3.89	5.93	0.174	0.699	5.70	429 ± 5	570 ± 20	37 ± 31
2	0.090	0.10	4.19	4.85	0.296	0.333	9.70	170 ± 4	353 ± 3	ND
3	0.108	0.13	3.83	4.84	0.203	0.555	6.71	318 ± 7	549 ± 4	ND
4	0.079	0.10	3.53	4.97	0.222	0.293	7.05	163 ± 1	369 ± 7	ND
5	0.075	0.09	3.27	4.37	0.166	0.409	4.61	317 ± 7	464 ± 18	ND
6	0.132	0.17	3.75	4.61	0.245	0.353	6.93	1018 ± 6	2021 ± 6	26 ± 44
7	0.094	0.13	3.92	4.66	0.241	0.411	7.16	499 ± 5	1025 ± 8	ND
8	0.088	0.12	3.49	4.48	0.197	0.219	5.50	569 ± 27	1175 ± 31	43 ± 74
9	0.156	0.13	3.30	4.11	0.252	0.781	5.98	1001 ± 13	1346 ± 21	ND
MID-STALK										
0	0.006	<.04	1.60	5.56	0.247	0.255	8.46	23 ± 2	51 ± 6	ND
1	0.010	<.04	2.48	7.04	0.215	0.737	7.79	50 ± 2	106 ± 2	ND
2	0.040	0.04	2.38	5.65	0.252	0.378	8.14	116 ± 10	353 ± 24	ND
3	0.071	0.07	2.17	5.48	0.244	0.480	9.11	183 ± 18	462 ± 3	ND
4	0.060	0.10	2.25	4.77	0.263	0.480	8.10	191 ± 5	435 ± 11	18 ± 30
5	0.061	0.08	2.31	4.51	0.207	0.705	5.57	310 ± 13	406 ± 25	ND
6	0.095	0.13	2.40	4.97	0.235	0.294	7.26	1208 ± 12	2430 ± 30	84 ± 2
7	0.106	0.12	2.20	4.65	0.217	0.268	7.14	279 ± 9	660 ± 15	ND
8	0.055	0.11	3.53	4.42	0.226	0.260	7.45	390 ± 13	821 ± 8	54 ± 47
9	0.122	0.12	2.36	4.27	0.248	1.158	5.41	1409 ± 3	1259 ± 11	67 ± 55
UPPER-STALK										
0	0.010	<.04	1.09	3.88	0.142	0.141	5.06	26 ± 4	39 ± 2	ND
1	0.008	<.04	1.54	4.01	0.099	0.912	5.01	28 ± 2	37 ± 2	ND
2	0.020	<.04	1.37	3.16	0.102	0.239	3.78	40 ± 2	91 ± 3	ND
3	0.036	<.04	1.34	2.66	0.095	0.433	4.32	101 ± 6	120 ± 11	ND
4	0.038	0.04	1.50	3.76	0.183	0.439	5.53	107 ± 3	148 ± 15	ND
5	0.042	0.05	1.51	3.79	0.161	0.549	4.92	132 ± 11	145 ± 3	26 ± 43
6	0.067	0.09	1.74	3.64	0.127	0.244	4.80	302 ± 3	495 ± 6	15 ± 24
7	0.055	0.07	1.40	3.14	0.106	0.308	3.62	291 ± 9	167 ± 7	ND
8	0.031	0.06	1.84	3.20	0.111	0.155	3.98	260 ± 12	361 ± 19	21 ± 36
9	0.096	0.07	1.63	3.25	0.148	0.545	3.89	479 ± 7	307 ± 11	ND

[a]Obtained by HPLC
[b]Values from AR using autoanalyzer method

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The analytical data obtained from the untreated tobacco are shown in Table 16. The values in the table were corrected to dry weight basis by use of the OV values obtained on the freeze-dried, ground, desiccated samples. No nitrite was detected in any of these samples. The nitrate-nitrogen values obtained by the HPLC technique were in reasonably good agreement with those reported by the AR Division, considering the differences in the methods.

Figure 22 shows the general upward trend in the nitrate values at each stalk position as a function of increasing curing time. Plots of nitrate-nitrogen/mg calcium also showed this trend, indicating that the gain in nitrate was not due to losses in constituents comprising dry weight. The data obtained in 1984 when primed burley leaves were air-cured did not show this increase, however, possibly indicating that nitrate in the stalks migrated to the lamina during air-curing. Nitrate values were considerably lower than normal for burley tobacco, due to the wet weather, delayed harvest, and possibly a combination of the soil in which the tobacco was grown and the amount of fertilizer applied.

Nornicotine and nicotine levels at each stalk position were extremely variable as a function of curing time, as shown in Figures 23-25, but anatabine was almost constant. In many cases, the variations were in opposite directions on a given week. This may be indicative of differences in nicotine to nornicotine conversion. As was true for nitrate, plots of the data after being normalized to the calcium values still gave essentially the same curves.

The nicotine values obtained by the HPLC method for minor alkaloids/nicotine were considerably higher than those reported by AR for

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FIGURE 23.
ALKALOIDS IN BURLEY DURING CURING

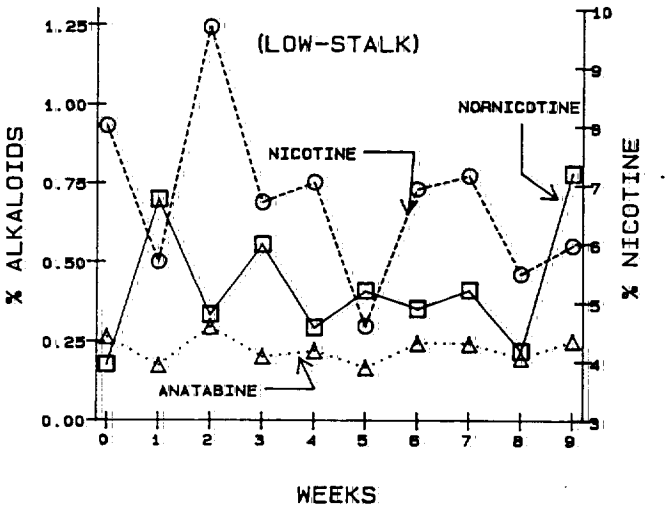


FIGURE 24.
ALKALOIDS IN BURLEY DURING CURING

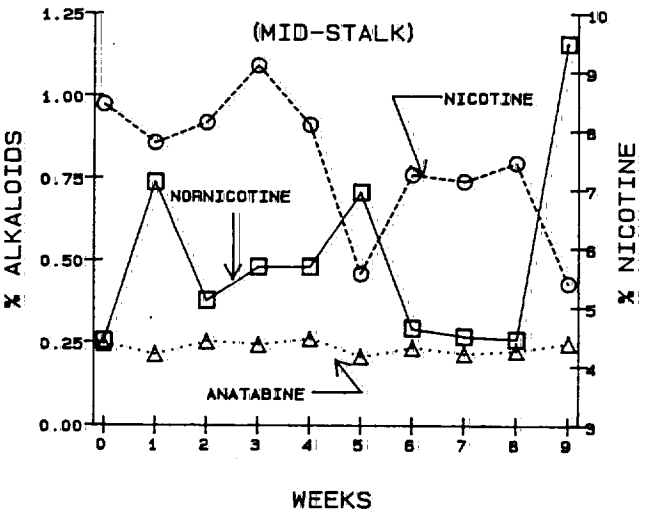
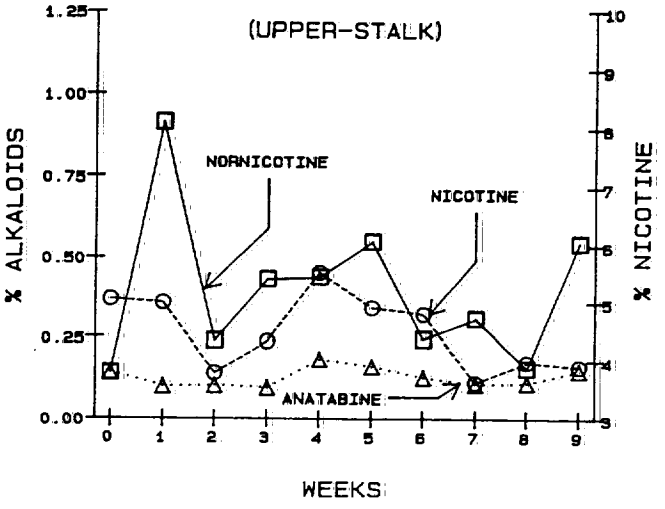


FIGURE 25.
ALKALOIDS IN BURLEY DURING CURING



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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

FIGURE 26.
TOTAL ALKALOIDS AND NICOTINE/Mg Ca

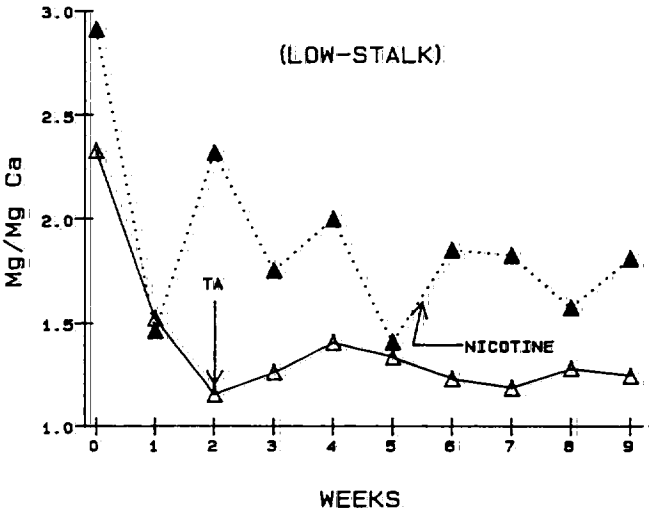


FIGURE 27.
TOTAL ALKALOIDS AND NICOTINE/Mg Ca

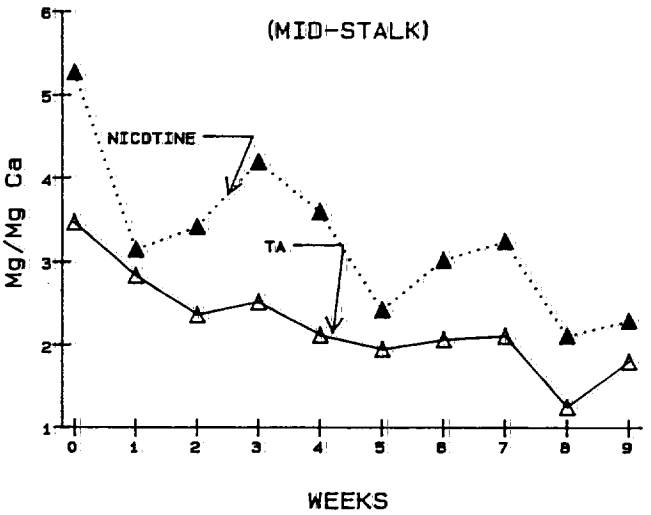
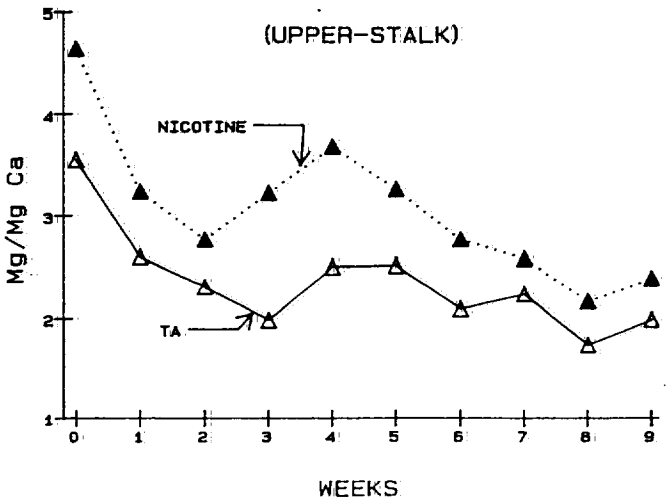


FIGURE 28.
TOTAL ALKALOIDS AND NICOTINE/Mg Ca



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total alkaloids. Figures 26-28 show the data compared after normalization to constant calcium content. Except for a few points, the two sets of curves followed essentially the same downward trend, with a constant difference in absolute amount. This difference may be due to a constant error in the HPLC method, although comparison of standard solutions prepared at different times showed no difference, and the linearity of the method over the range of the measurements was excellent. Part of the error may be due to a tendency for total alkaloid values to be lower than similar values obtained by other methods. Since the differences were only in absolute terms, no further efforts were made to find the reasons for the descrepancies.

Plots of the increases in TSNA as a function of curing time are shown in Figures 29-31. These increases appeared to be almost linear beginning with the first week, except for Week 6, where NNN and NAT both increased to the highest level of the study at all three stalk positions. There does not appear to be any rational explanation for this anomaly other than a constant error that may have occurred during the analysis of the 6th week samples. Plots of the TSNA data after normalization for calcium content showed essentially the same curves as in Figures 29-31, and are, therefore, not included in this report.

The relationship of alkaloid content and nitrate content to the levels of individual TSNA was determined by calculating correlation coefficients between columns of data shown in Table 16. The nitrate, nornicotine and nicotine data columns were compared to the NNN and NAT columns. The correlation coefficients are shown in Table 17. The nitrate levels were correlated with NNN and NAT, but the alkaloids were not correlated. This simply indicates that the increase in TSNA during

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FIGURE 29.
TSNA FORMATION IN BURLEY

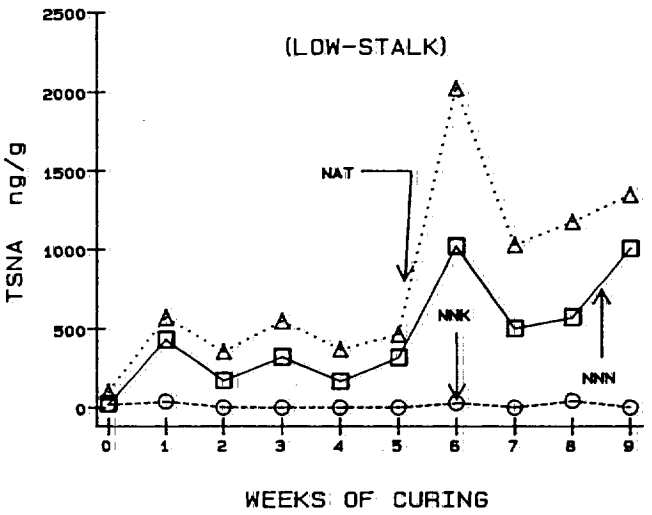


FIGURE 30.
TSNA FORMATION IN BURLEY

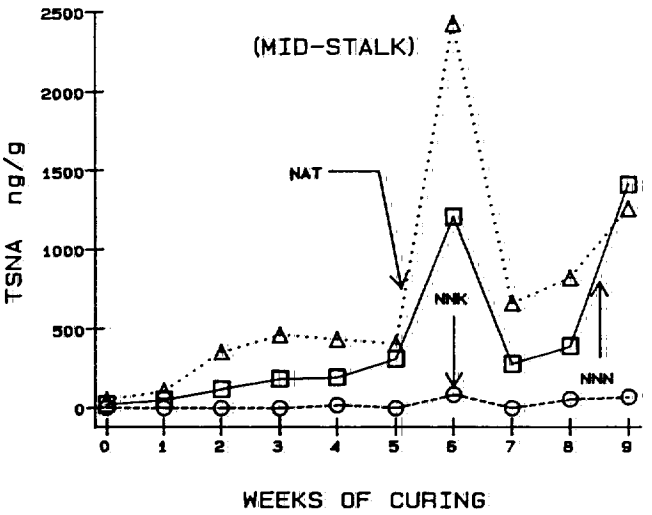
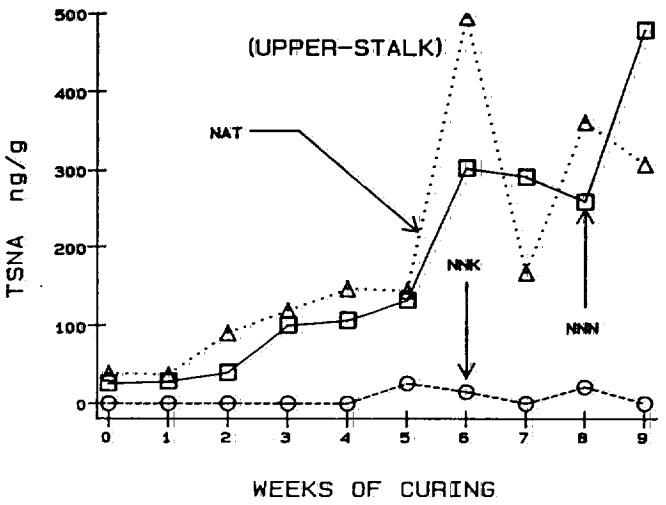


FIGURE 31.
TSNA FORMATION IN BURLEY



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curing paralleled the increase in nitrate. Since the alkaloids did not increase during curing, there was no correlation with the increase in TSNA. This does not imply that the alkaloids were not the precursors of the TSNA. However, the data suggest that the rate limiting reagent is dependant on the nitrate levels available at a particular time.

TABLE 17.
CORRELATION COEFFICIENTS FOR UNTREATED BURLEY.
Calculated by comparing each pair of data values as a function of sampling time.

CORRELATION	STALK POSITION		
	LOW	MID	UPPER
NO_3^--N VS NNN	0.741	0.753	0.923
NO_3^--N VS NAT	0.710	0.671	0.676
NIC VS NNN	-0.366	-0.617	-0.483
NORNIC VS NNN	0.464	0.441	-0.106
ANAT VS NAT	0.054	0.031	0.079

The stalks are known to contain high levels of nitrate, and NRA is believed to exist in the stalks long after the leaf levels have disappeared. In view of the fact that nitrate appears to migrate from the stalks to the leaves during air-curing, it is feasible that nitrite also migrates in this manner. The levels in this study were too low for detection, but possibly still high enough to cause TSNA formation.

Table 18 shows the microbiological data determined on the upper-stalk lamina during the curing study[41]. Protein values were determined in order to allow expression of NRA/mg protein. No NRA was detected, however, and no column for these data was included. The protein values show that protease activity was essentially complete after the first week of curing. Bacterial cell counts were measured on the leaf surfaces using both nutrient agar and tobacco agar as culture media. The data

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obtained using nutrient agar showed no real changes with increasing curing time other than sample-to-sample fluctuations. There was a slight decrease in bacteria with time when tobacco agar was used. The preparation of tobacco agar has been described. This medium was devised as an attempt to simulate the tobacco environment more closely than is possible with nutrient agar. The organisms generally exhibit less growth on tobacco agar than on nutrient agar, as seen in Table 18.

TABLE 18.
BIOLOGICAL DATA FROM BURLEY CURING STUDY
(Untreated)

WEEKS	OV	mg PROTEIN per g DW	CELLS/g Dry Weight NUTR AGAR	CELLS/g Dry Weight TOB AGAR
0	81.73	72.76	2.80e+06	4.49e+05
1	63.12	27.83	1.89e+06	1.93e+05
2	34.20		3.10e+07	2.36e+05
3	24.26	24.90		
4	45.60	29.34	6.38e+06	2.65e+05
5	19.48	25.92	2.10e+06	2.90e+05
6	16.29	27.42	3.05e+07	3.70e+04
7	16.00	20.84	3.20e+06	1.10e+04
8	16.77	28.38	2.20e+06	5.30e+03

In Table 19 the data obtained from the analysis of the treated tobacco are presented. The data for untreated tobacco are also included for comparison at each point and stalk position where the treated tobacco was sampled. To visualize the effects of the treatments more easily, bargraphs were prepared. In Figures 32-34, the effects on alkaloid levels after 9 weeks of curing are shown at all three stalk positions. The nicotine values are shown after dividing by 10 to allow all the data to be presented on the same scale. The only apparent effect of the treatments on alkaloids is a reduction in normicotine in all the treated samples relative to the untreated after 9 weeks. This effect can be explained by referring to Figures 23-25, which show that in all the

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TABLE 19. BURLEY CURING STUDY, TREATED.

Treatments as follows: a=untreated; b=Vitamin E Acetate; c=Vitamin C Palmitate; d=Vitamin E Acetate + Sodium Ascorbate; e=Tween 80 Control. All data reported on dry weight basis.

TREATMENT	WEEKS	%NO_3^--N	%COT	%MYOS	%ANAT	%NORN	%NIC	TSNA, ng/g (± SD)		
								NNN	NAT	NNK
LOW-STALK										
a	0	0.011	0.0049	ND	0.263	0.175	8.01	23 ± 3	100 ± 6	16 ± 26
a	1	0.024	0.0054	0.0053	0.174	0.699	5.70	429 ± 5	571 ± 20	37 ± 31
b	1	0.007	ND	ND	0.248	0.181	7.92	NA	NA	NA
c	1	0.013	0.0023	0.0019	0.105	0.394	7.42	NA	NA	NA
d	1	0.020	0.0118	0.0036	0.265	0.228	8.86	NA	NA	NA
e	1	0.052	0.0277	0.0022	0.199	0.762	8.06	NA	NA	NA
a	9	0.156	ND	0.0054	0.252	0.781	5.98	100 ± 13	1346 ± 21	ND
b	9	0.100	ND	0.0023	0.235	0.209	7.12	421 11	1241 24	35 ± 59
c	9	0.075	ND	0.0023	0.249	0.207	6.72	270 ± 7	915 ± 34	58 ± 56
d	9	0.098	ND	0.0022	0.217	0.276	5.55	491 ± 5	1243 ± 30	59 ± 54
e	9	0.158	ND	0.0028	0.199	0.386	4.66	882 ± 10	1415 ± 12	60 ± 52
MID-STALK										
a	0	0.006	0.0224	ND	0.247	0.255	8.46	23 ± 2	51 ± 6	ND
a	1	0.010	ND	0.0047	0.215	0.737	7.79	50 ± 2	106 ± 2	ND
b	1	0.016	ND	ND	0.298	0.246	9.67	ND ±	160 ± 6	ND
c	1	0.006	ND	0.0021	0.292	0.701	9.44	63 ± 6	119 ± 9	ND
d	1	0.008	0.0026	0.0017	0.288	0.598	9.76	48 ± 4	84 ± 4	ND
e	1	0.012	ND	0.0037	0.027	0.827	7.49	90 ± 3	46 ± 2	ND
a	9	0.122	ND	0.0068	0.248	1.158	5.41	1409 ± 3	1260 ± 11	67 ± 55
b	9	0.115	ND	0.0027	0.239	0.226	7.79	396 ± 13	1270 ± 19	72 ± 28
c	9	0.103	ND	0.0049	0.171	0.460	4.97	734 ± 9	870 ± 22	37 ± 62
d	9	0.096	ND	0.0042	0.219	0.741	5.68	888 ± 6	1093 ± 41	81 ± 11
e	9	0.131	ND	0.0031	0.236	0.430	6.04	742 ± 13	1658 ± 14	139 ± 11
UPPER-STALK										
a	0	0.010	0.0219	ND	0.142	0.141	5.06	26 ± 4	39 ± 2	ND
a	1	0.008	ND	0.0009	0.099	0.912	5.01	28 ± 2	37 ± 2	ND
b	1	0.010	0.0018	ND	0.174	0.358	5.52	NA	NA	NA
c	1	0.008	ND	0.0033	0.068	0.604	5.18	NA	NA	NA
d	1	0.006	0.0023	0.0018	0.119	0.515	5.67	NA	NA	NA
e	1	0.009	ND	0.0051	0.067	0.868	3.85	NA	NA	NA
a	9	0.096	ND	0.0040	0.148	0.545	3.89	479 ± 7	307 ± 11	ND
b	9	0.065	ND	0.0022	0.154	0.216	4.74	458 ± 18	877 ± 29	34 ± 57
c	9	0.046	ND	0.0027	0.167	0.326	4.71	242 ± 3	264 ± 12	28 ± 44
d	9	0.055	ND	0.0034	0.147	0.447	4.54	339 ± 10	510 ± 6	ND
e	9	0.101	ND	0.0036	0.109	0.523	3.34	335 ± 15	291 ± 9	26 ± 42

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untreated samples, the levels of nornicotine were significantly higher in the 9th week than in the preceeding weeks. It is doubtful that this effect was real. More likely, it is due to an analytical variation that occurred when the untreated samples from the 9th week were being analyzed.

Figures 35-37 show the effects of the treatments on TSNA levels after 9 weeks of curing at all three stalk positions. The upper-stalk TSNA values were low, and the results at this stalk position were not very meaningful, presumably due to lower accuracy in the determination. The NAT values at the lower and middle stalk positions were lowest for the ascorbyl palmitate-treated tocacco. The effects of treatment on NNN values were less definite. The data do not appear to show appreciable effects of these treatments in general. This may be because of the paucity of data points, the decomposition of the antioxidants, or the lack of effectiveness of the treatments.

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FIGURE 32.
EFFECT OF TREATMENTS ON
BURLEY CURED 9 WEEKS
(LOW-STALK)

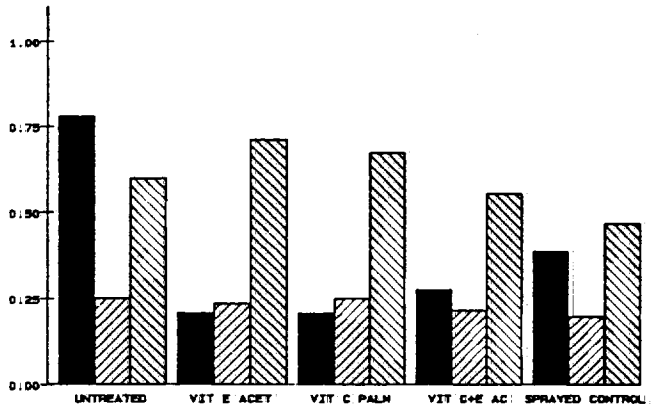


FIGURE 33.
EFFECT OF TREATMENTS ON
BURLEY CURED 9 WEEKS
(MID-STALK)

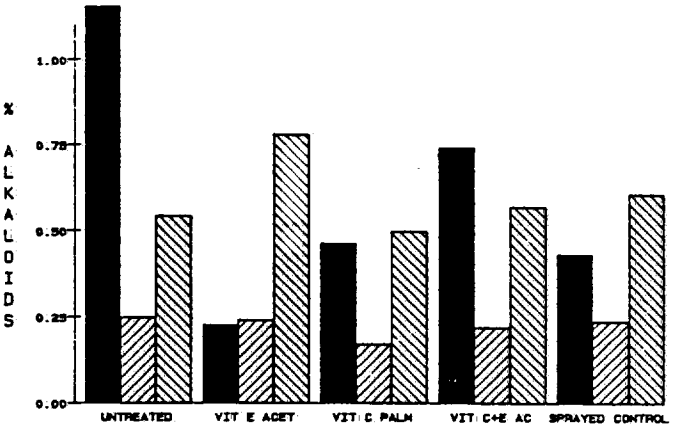
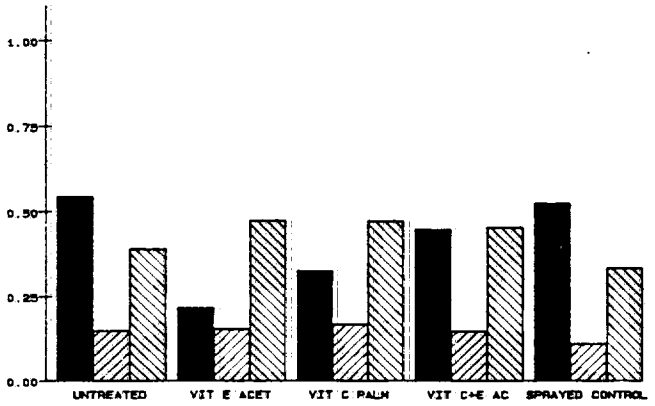


FIGURE 34.
EFFECT OF TREATMENTS ON
BURLEY CURED 9 WEEKS
(UPPER-STALK)



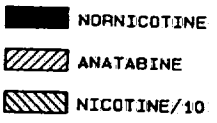


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FIGURE 35.
EFFECT OF BURLEY TREATMENTS ON
TSNA AFTER 9 WEEKS CURING
(LOW-STALK)

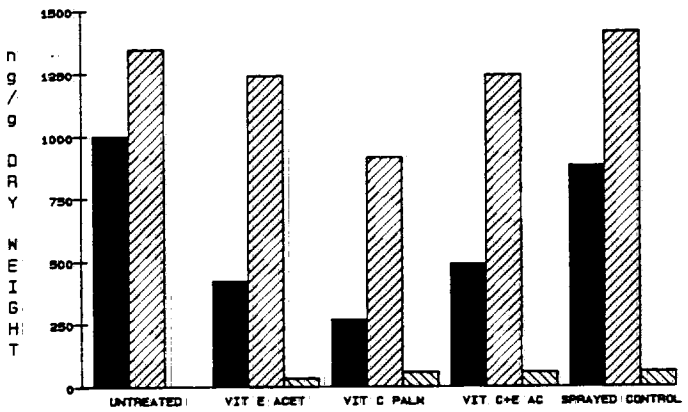


FIGURE 36.
EFFECT OF BURLEY TREATMENTS ON
TSNA AFTER 9 WEEKS CURING
(MID-STALK)

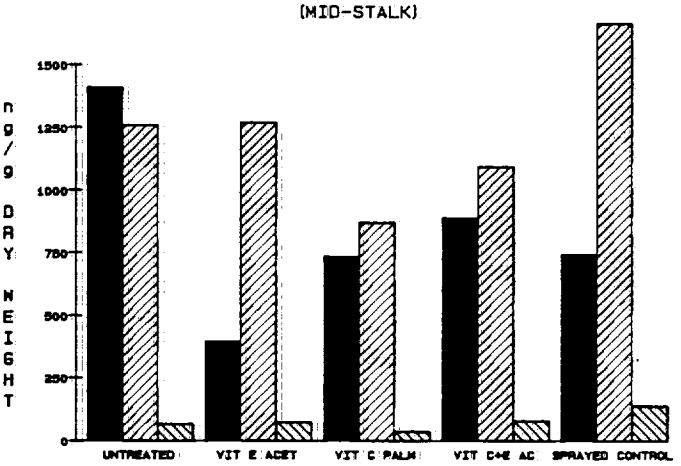
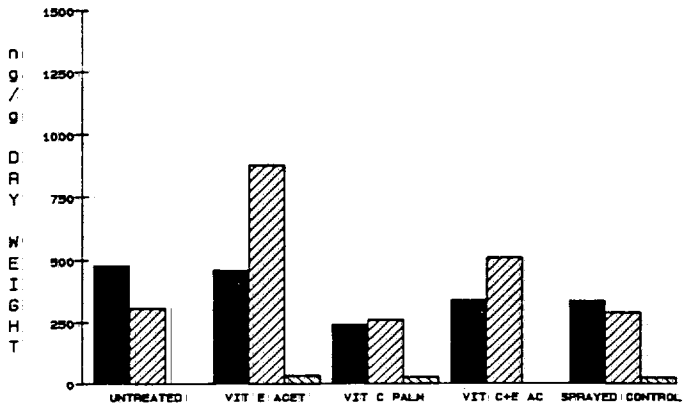


FIGURE 37.
EFFECT OF BURLEY TREATMENTS ON
TSNA AFTER 9 WEEKS CURING
(UPPER-STALK)



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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

SUMMARY OF DATA FROM BURLEY CURING STUDY

1. No NRA was detected in the lamina throughout the study.
2. No nitrite was detected.
3. Protein values were constant after the first week.
4. There was no increase in bacterial cell counts during air-curing.
5. Nitrate increased during curing as a function of time.
6. Alkaloids changed little with time, but there were large fluctuations due to sample variations, local variations in nicotine-nornicotine conversions, or both.
7. TSNA increased as a function of time, but there were larger increases in the 6th week.
8. TSNA were three times as high in the lower and middle stalk position leaves after curing than in the upper-stalk leaves.
9. There was some lowering of NNN and NNK in the lower and middle-stalk leaves due to ascorbyl palmitate and tocophorol acetate, although the results were not dramatic.

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

C. Present State of Knowledge on Formation of TSNA in Filler

Air-curing: In air-curing, the ultimate precursors of the TSNA are the alkaloids and nitrate. Nitrate must be converted to nitrite by either enzymatic or chemical means. Nitrite is generated via NR during the green growth stage, and this may interact with solanesol or other terpenes to form nitrite esters, etc. These organic NSA may interact with alkaloids during curing to form TSNA. Another possibility is free radical nitrosation. Nitrate is apparently transferred into the lamina from the stalks during curing, and there is reason to believe that nitrite may be transferred as well. Bacteria can reduce nitrate, and in some cases can catalyze nitrosamine formation. Although they are undoubtedly important in the formation of TSNA in HLC and fermented tobaccos, their importance in air-curing has not been demonstrated.

Most air-curing studies have reported correlations between nicotine and NNN. Nornicotine appears to be correlated as well. The immediate precursor of NNN appears to be nornicotine, although it has been shown that NNN can also be formed from nicotine-N'-oxide[42]. NNK can be derived from nicotine at low rates, but a more likely precursor is pseudo-oxynicotine (the non-nitrosated amine corresponding to NNK). This compound is formed from nicotine, and is in equilibrium with N-methyl myosmine[43]. No reports of the isolation of pseudo-oxynicotine from tobacco have appeared, possibly due to its instability.

Flue-Curing:

In flue-curing, much less information is available. The precursors of TSNA are probably essentially the same as in air-curing and the same mechanisms are possible, but it is likely that different mechanisms are predominant. NRA is not detectable, nor is nitrite. Since leaves are primed, the nitrate level is fixed, and there is no chance for nitrite to be

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transfered into the lamina. This makes the organic NSA mechanism more likely, especially since the increase in temperature would increase the rate of the reaction. Free radical mechanisms are more likely for the same reason. Results show no correlation of TSNA with alkaloids in flue-curing. The distribution of TSNA in the leaves is very different from that found in air-curing, where the TSNA tend to be correlated with the position of nitrate; i.e., more concentrated in the midvein and central portions of the leaf[44]. There is no correlation of TSNA with nitrate, although low nitrate tends to yield far lower TSNA levels[45].

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

D. Plans for 1986

1. CHEMICAL MECHANISMS OF TSNA FORMATION IN FILLER

A. ORGANIC NSA: Having established the possibility that organic NSA can be formed from tobacco wax, experiments are planned to determine whether this reaction actually occurs in tobacco. A method will be worked out to extract these NSA out of tobacco and determine their relative capability to nitrosate amines under non-aqueous conditions. This method will then be applied to tobacco samples available from the curing studies and from green tobacco sampled in the field. Later attempts may be made to identify the organic NSA if positive results are obtained in the first part of the study.

B. ROLE OF PSEUDO-OXYNICOTINE: Samples of pseudo-oxynicotine will be obtained if possible, and model reactions with nitrite carried out to determine whether NNK is formed. If successful, plans will be made to look for this compound in tobacco.

C. ROLE OF NICOTINE-N'-OXIDE: The reaction of this compound with nitrite will be attempted to determine whether it may be a possible precursor of NNN. Further attempts may be made to measure the oxide in tobacco, and determine whether this pathway is involved during curing.

2. BIOCHEMICAL MECHANISMS OF TSNA FORMATION

A. IMMUNOLOGICAL METHODS FOR NR: A polyclonal antibody for squash NR will be used to demonstrate the presence or absence of the enzyme in situ using greenhouse tobacco[46].

B. LEAF STERILIZATION STUDIES: A further attempt will be made to sterilize tobacco leaves and cure them in a sterile environment in order to establish the role of microorganisms in TSNA formation.

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Source: https://www.industrydocuments.ucsf.edu/docs/mxbl0000

C. MICROBIAL ENZYMES: Organisms will be selected from tobacco and characterized. These organisms will be examined for the presence or absence of NR[47].

D. IMMUNOCYTOCHEMICAL STUDIES OF NR: Monoclonal antibodies will be prepared to plant and microbial NR. The antibodies will be used to locate and identify the enzymes in situ during curing and possibly aging[47]. This will be done only if the immunological results dictate it.

3. PHYTOTRON STUDIES[48]

The following studies have been planned to take place on tobacco plants growing in growth chambers at the Phytotron, a facility located at NC State University.

A. EFFECT OF FERTILIZATION/WATERING SCHEDULE ON BURLEY TSNA: The effect of establishing a high-nitrate fertilizer regime during the growth period, followed by a rapid leaching of the soil nitrate before and after topping will be determined. This should allow determination of whether the organic NSA mechanism takes place as postulated.

B. DETERMINE OF WHETHER TSNA FORM DURING CURING IN THE ABSENCE OF NITRATE: Burley plants will be grown in sterile soil using only amino nitrogen, and the plants will be air-cured. TSNA will be determined to establish whether or not formation occurs in the absence of nitrate in the plants.

4. CURING STUDIES

A. BRIGHT, FLUE-CURING STUDY[49]: A further bright curing study has been planned for 1986, since the 1985 study failed to answer many questions on formation of TSNA during flue-curing. The plants will be grown at the VPI experimental station in Blacksburg, VA. In addition to the time-course data obtained previously, the tobacco will be sampled at

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various stages of growth to learn more about the organic NSA formation, and the methods described under 1.A. will be applied on samples from the different stages of curing to determine whether the organic NSA mechanism is operative. The effect of treatment with antioxidants will be determined as well.

B. BURLEY, AIR-CURING STUDY[50]: A burley air-curing study will be carried out again in 1986. The objectives will be the same as in 1985, with more emphasis on learning about the mechanisms of nitrosation, as outlined above. Also, comparisons of stalk-curing and primed curing methods will be made. This is being included to examine more closely the observation that nitrate and possibly nitrite are transferred into the leaves from the stalks. In addition, the antioxidant treatments will be repeated.

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